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TESTUDINID HERPESVIRUS 3: DETECTION AND MOLECULAR CHARACTERIZATION OF STRAINS IN ITALIAN *TESTUDO* SPP.

Tesi di dottorato di:
Dr. Marco TECILLA
R10820

TUTOR: Prof.ssa Paola Roccabianca (University of Milan, Italy)

CO-TUTOR: Prof. Francesco Origgi (University of Bern, Switzerland)

COORDINATORE: Prof. Fulvio Gandolfi

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English abstract.

Herpesvirales is an ancient group of DNA viruses that infect different animal species, including reptiles. The *Alphaherpesvirus* is the major subfamily of Herpesvirales involved in reptile infections, and responsible for the onset of variably severe necrotizing lesions, affecting different body parts, and frequently associated with the animal's death. The death of the animal can be caused directly by the effect of the virus, or more frequently by the onset of secondary bacterial infections. Today, the reptile species most commonly kept as household pets are tortoises, lizards, and snakes. Compared to the owners of the other two groups of reptiles, at least in Europe, pet tortoise owners frequently buy animals that are collected from the wild (especially exotic species) and are also prone to abandon these animals into the wild. Moreover, good breeding practices are frequently not applied to reptile breeding centers, which commonly introduce new animals in their enclosure without testing and/or quarantining the animals. These habits have different consequences, including spreading both exotic and non-exotic diseases on Italian soil. Among all viruses infecting chelonians, herpesviruses are the group with the highest number of members, with more than five groups affecting turtles and tortoises. International literature reports herpesviruses as one of the most relevant causes of death in chelonians. Despite the relevance of the disease from both a conservational and economical point of view, no information is available on the presence of these viruses in Italy, and very little is known about the genetic characteristics and the host-pathogen interaction mechanisms. This work aims to identify the presence of Testudinid herpesviruses (TeHVs) in Italy, characterize the genome of the virus, and start to understand the host-pathogen interaction mechanisms between TeHV3 and *Testudo graeca*. To evaluate the presence of TeHVs in Italy, we carried out a prospective study, performing the ELISA test on live animals in the WWF Vanzago's Oasis, combined with PCR on both prospective and retrospective samples collected from the archive of the anatomical pathology section of the University of Milan. Complete necropsy and histological evaluation were performed on all prospective samples after their natural death. Furthermore, viral isolation was successfully carried out on one of the Vanzago cases. Using both TeHV3 strains collected in Italy, USA, and Switzerland, complete genome sequencing was performed. Evaluation of *T. graeca* immune response against TeHV3 was evaluated by creating a bacteriophage expression library screened with hyperimmune tortoise sera, obtained from a previous transmission study.

Our study demonstrates that from both the prospective and retrospective samples, all that were positive were TeHV3, but one retrospective sample was TeHV1 positive.

TeHV3 genome sequencing demonstrated that the viral genome is at least 150,080 nucleotides long, arranged in a D-type configuration, and extensively co-linear with the human herpesvirus 1 genome. From an immunological point of view, we were able to identify three relevant TeHV3 candidate antigenic proteins, TE-17, UL-15, and gB. Although we initially supposed the gB was the most relevant antigenic protein in *T. graeca* immune response against TeHV3, further investigation showed that the gB sequence in the evaluated phagemid was antisense compared to the origin of replication, and could not be transcribed. To assess the possible immunological relevance of TeHV3 gB protein in boosting host immune response, we performed enzymatic restriction, PCR cloning and PCR direct site mutagenesis on the original phagemid, to obtain new structures containing only one of the three possible immunogenic proteins previously identified.

Despite the numerous attempts and techniques used, we are currently not able to obtain viable bacteria to confirm our hypothesis.

Abstract in Italiano.

Gli Herpesvirales sono un gruppo di virus a DNA ontogeneticamente antico, in grado di infettare diverse specie animali inclusi i rettili. Tra le diverse famiglie di herpesvirus gli *Alphaherpesvirus* sono la sottofamiglia di Herpesvirales maggiormente coinvolta nelle infezioni di rettili e sono responsabili di lesioni necrotizzanti di gravità variabile a carico di diversi organi; spesso le lesioni portano a morte il soggetto sia a causa dei danni indotti direttamente dal virus che per l'insorgenza di infezioni batteriche secondarie. Tra tutte le specie di rettili le tartarughe, le lucertole e i serpenti sono gli animali domestici più comuni. I proprietari di tartarughe sono, tra le tipologie di proprietari di rettili, quelli più proni ad acquistare animali prelevati in natura e ad abbandonare specie alloctone sul territorio. Inoltre, sono presenti numerosi allevamenti amatoriali di queste specie che non rispettano le buone norme di allevamento e introducono animali senza effettuare regolare quarantena. La somma di questi comportamenti ha avuto molteplici conseguenze, tra le quali la diffusione di malattie esotiche e non esotiche nelle popolazioni autoctone di cheloni.

Tra tutti i virus che possono causare infezioni nelle tartarughe, gli Herpesvirus sono i maggiormente rappresentati, con 5 gruppi in grado di infettare testuggini terrestri e acquatiche. Inoltre, sulla base di quanto riportato in letteratura, gli herpesvirus sono una delle principali cause di morte tra i cheloni. Sebbene questi agenti eziologici abbiano un ruolo di rilievo sia da un punto di vista della bioconservazione che economico, non sono presenti informazioni sulla presenza del virus in Italia e, in linea generale, pochissime informazioni sono disponibili sulle caratteristiche genetiche e sui meccanismi di interazione ospite-patogeno.

Questo lavoro ha lo scopo di identificare la presenza di *Testudinid herpesvirus* (TeHVs) in Italia, caratterizzare il genoma del virus ed iniziare ad investigare i meccanismi di interazione ospite-patogeno tra TeHV3 e *Testudo graeca*.

Per valutare la presenza dei TeHVs in Italia, abbiamo effettuato uno studio prospettico effettuando test ELISA e PCR su tutte le tartarughe presenti nell'Oasi WWF di Vanzago ed abbiamo fatto, inoltre, uno studio retrospettivo effettuando la PCR sui campioni d'archivio dell'Istituto di Anatomia Patologica Veterinaria dell'Università di Milano. Esame necroscopico ed istopatologico completo sono stati effettuati su tutti i soggetti deceduti spontaneamente provenienti dall'Oasi di Vanzago. Inoltre, da uno dei soggetti provenienti dall'Oasi è stato possibile effettuare l'isolamento virale. Utilizzando ceppi di TeHV3 provenienti da Italia, USA e Svizzera, è stato possibile sequenziare il genoma di TeHV3. La valutazione dell'interazione ospite-patogeno è stata fatta utilizzando una libreria fagica screenata con siero iperimmune di *T. graeca*, ottenuto da uno studio di trasmissione precedente.

Il nostro studio ha dimostrato che tutti i campioni positivi, sia prospettici che retrospettivi, erano positivi per TeHV3, tranne un caso retrospettivo positivo per TeHV1.
Il genoma di TeHV3 è risultato essere lungo, circa, 150.080 nucleotidi, con una configurazione genomica di tipo D ed è risultato altamente co-lineare con il genoma dell'*Human herpesvirus 1*.
Da un punto di vista immunologico, abbiamo individuato tre potenziali proteine responsabili della risposta immunitaria dell'ospite, TE-17, UL-15 e la gB. Sebbene si fosse inizialmente ritenuto che la gB fosse la principale proteina responsabile della risposta immunitaria, valutazioni successive hanno dimostrato che l'orientamento della sequenza che codifica per la gB nel fagemide fosse antisenso e che, quindi, non potesse essere trascritta. Per valutare il possibile ruolo antigenico di gB, il fagemide originale è stato modificato in modo tale da contenere un solo frammento codificante. Le tecniche utilizzate per modificare il fagemide sono state: restrizione enzimatica, clonaggio con PCR e *Site-direct mutagenesis* con PCR.
Sebbene siano stati fatti numerosi tentativi e utilizzate metodologie differenti, a tutt'oggi, non è stato possibile ottenere colonie batteriche contenti il fagemide modificato e, quindi, non è stato possibile confermare l'ipotesi originale.

Summary of the work.

Numerous infectious diseases have been documented in reptiles, however there is very little information about their immunological response. One of the most diffuse and lethal reptile pathogens is *Testudinid herpesvirus 3* (TeHV3), an *Alphaherpesvirinae*. All species of tortoises (*Testudinidea)* are considered susceptible to TeHV3, however the virus is over represented in the genus *Testudo*, which includes, among others, *T. graeca*, *T. hermanni*, *T. marginata*, and *T. horsfieldii*, which are popular pets in Europe. The incidence of TeHV3-associated disease is highest right after hibernation.

The aim of this work is to partially characterize the immunological response of *T. graeca* to TeHV3. A bacteriophage library composed of about 5,000 clones containing genomic DNA fragments of TeHV3 was produced. Bacteriophages were amplified in a specific strain of E. coli and screened with TeHV3-seropositive sera from *T. graeca*. Phagemids were excised from the positive bacteriophages, sequenced, and compared with the TeHV3 genome to identify the encoding genes. Six different structural and non-structural proteins were identified as immune relevant. Vero cells were transfected with phagemids of the positive clones to confirm the previous results. TeHV3's protein expression was assessed by F.A.C.S using *T. graeca* seropositive sera. Out of all six selected clones, only that expressing the partial sequence of the glycoprotein B (gB) showed a positive signal in the F.A.C.S. analysis. This result is consistent with the well-known immunogenicity of gB of other herpesviruses, including those infecting humans, and with the highly conserved role that gB plays in host-pathogen interaction across species and evolution.

Scientific background and working hypothesis:

Italy is one of the European countries with the highest population of pet and wild tortoises. From the early 90s reptiles, that were once mostly considered as wild animals, have been integrated in households, gaining the status of "non-conventional pets." Despite this interest, Italian *Testudo* sp. population has steadily and rapidly declined in the last 20 years. Not only free ranging tortoises are in danger, but also pet and breeding animals have shown a progressive reduction in survival time, and the annual birth rate has been dramatically reduced, with severe economic losses. The causes of these losses have been hypothesized but not consistently evaluated, and have been ascribed to the emergence of infectious diseases and a severe increase of environmental pollution. The percentage of endangered reptile species increased from 9.94% in 2008 to 22.2% in 2014, of these 168 are critically endangered, 349 are endangered and 395 are vulnerable according to the World Conservation Union (IUNC) Red List. More specifically,

the IUCN Red List reports 6 species as extinct, 31 as critically endangered, 44 as endangered and 59 as vulnerable on a global scale. Most of these species are imperilled by excessive harvesting and loss of suitable habitats [44], but recent work on environmental toxicology of aquatic species has shown that toxicants and pollution can seriously influence the reproduction activity and embryonic development of reptiles [35, 37]. In Italy the IUNC Red List reports four species of reptile in danger, among which are *Testudo hermanni* ["near threatened" class] and *Testudo graeca* ("vulnerable" class). These species, with the addition of the *T. marginata*, are the most represented in Italy and they are also included in the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) appendices II (species that are not necessarily now threatened by extinction but that may become so unless trade is closely controlled). Regarding the specific reason for the progressive decline of these species, especially during the last 30 years, there are seven most frequently mentioned causes: habitat loss [7, 11, 35, 36, 38, 39], removal from original habitat [6, 39], environmental contamination [11, 35], climate change [11], invasive exotic species [11], disease and parasitism [11], cascading decline consequent to the numerical reduction of other related species [11].

One of the most relevant infectious diseases that threaten the global survival of the *Testudo* spp. is the progressive diffusion of the *Testudinid herpesvirus* [18]. Many effects of a chronic infection with the *T. herpesvirus* may mimic pollutant effects; this contributes to the confusion on the various causes of the population decline. Tortoises' and turtles' herpesviruses have been characterized recently and, as reported by ICTV (International Committee on Taxonomy of Viruses), have been temporarily classified in the α- Herpesvirus, proposed genera Chelonivirus. Included in this genus are two proposed species: *Testudinid herpesvirus* (TeHVs), usually infecting tortoise, and *Chelonid herpesvirus* [ChHV], usually infecting turtles [30, 47]. TeHVs are temporarily, divided into four genotypes. The origin of the four genotypes of TeHVs is not clear [30], but due to commercial trading they have a worldwide distribution. TeHV1 and TeHV3 [Eurasia] are both present in Europe [32, 45, 55] while TeHV2 has been identified in the USA and TeHV4 has been detected in Africa. TeHV3 is the best characterized genotype of *Testudinid herpesvirus* in Europe [19, 30]. All *Testudo sp.* species seem to be susceptible to the infection, but different levels of susceptibility have been hypothesized [18, 42]. TeHV3 infections are commonly defined by clinicians as "running nose" or "rhinitis- stomatitis complex" [30] but the clinical picture is more complex than the name would suggest. According to literature, the pathology starts as an upper respiratory-digestive tract disease with the involvement of nares [usually bilateral], eyes, gums and tongue [12, 30, 45, 55]. Macroscopically, the animal presents with a muco-purulent bilateral nasal discharge associated with a diphtheronecrotic sto-

matitis-glossitis whit mono-lateral or bilateral conjunctivitis [15, 30, 42, 45, 52, 55]. Microscopically, pneumonia has been observed, but the role of TeHV3 in the latter is not clear [30, 55]. Microscopic lesions related to the TeHV3 infection are mainly necrotizing with occasional syncytia [30, 31, 42] in the mucosal epithelium, lung and central nervous system. The single specific diagnostic finding for TeHV3 infection is the presence of the typical, intranuclear, amphophilic inclusion bodies [13, 55]. Diagnosis of TeHV3 is performed with the following tests:
1) Serology**:** It is an ELISA test, based on a colorimetric reaction. The test is now being validated for Geek's (*Testudo graeca*) and Hermann's (*Testudo hermanni*) tortoise with a sensitivity and a specificity of 97% and 98% respectively. The major limitation of this test is that it has not been validated on other tortoise species, moreover animals infected with the clinical disease have usually not yet seroconverted [19, 30, 31, 32, 55]
2) Serum-neutralization**:** This test has the same pros and cons as the ELISA [30, 31, 32].
3) Histology: This technique has low sensitivity but high specificity. Necrotic lesions in typical anatomical areas can be suggestive of TeHV3 infection, while the identification of typical inclusion bodies is diagnostic [30, 31, 32, 33, 55]. Unfortunately, it is mostly applied postmortem.
4) PCR: identifies viral DNA, but positivity is not directly related to disease due to the characteristic behavior of Herpesviruses to develop latency [30, 31, 48].
5) Viral isolation: tortoise heart cells (*Terrapente* heart cells, TH-1) are generally infected through direct contact with a purified and homogenized sample of a suspected animal tissue [30]. Viral pathogenicity can be affected by conservation procedures, because of this viral isolation requires long observation times. Despite time requirements, virial isolation should always be included in the "heard health" cases, to better complement viral characteristics and possibly develop a "in-house" vaccine based on the strain of the virus involved in the outbreak [30, 55].

Despite the awareness of the presence and the spread of the virus in the *Testudo* spp. population, the long latency period associated with the time needed to produce an effective antibody concentration is considered the most accredited cause of the spread of the virus and the severe loss of tortoises.

Aims:

1) Evaluate for TeHV3 presence in Italy. Little is known regarding the prevalence and the distribution of TeHV3 in Europe, especially in Italy. What we expect is to find a prevalence similar to those identified in Spain, the UK, and the USA.
We will also try to investigate the presence of TeHVs genotypes other than TeHV3.

2) Evaluate gross and microscopic lesions induced by TeHV3. Basing on the observation made in different countries about the pathological findings correlated to TeHV3 infections, we will try to confirm if the lesions reported in literature are also present in Italian tortoises, understand if TeHV3 induces different lesion profiles in different tortoise species, and if different strains of TeHV3 are present.

3) Start investigating the immunological response of tortoises against viruses. With a bacteriophage expression library, we would like to try to identify the most relevant immunogenic proteins of TeHV3 recognized by the host immune system. The purpose of this work is to better complement the host pathogen interaction, and try to develop new preventive approaches including vaccines and more specific diagnostic tests.

Sample collection:

Samples were sent to us from the Oasis of Vanzago, a WWF oasis located near Milan, and by veterinarians as routine diagnostic cases for the anatomical pathology service of the Faculty of Veterinary medicine of the University of Milan. A total of 18 tortoises were collected. Additional 10 cases, originating from the same geographical area, were obtained as routine diagnostic animals.
PCR and histopathology were performed on both groups, while necropsy and virological analyses (such as viral isolation) were only carried out on the samples.

Necropsy and prospective sample collections:

All the twenty-eight dead animals were submitted to the veterinary pathology section of DIVET for necropsy evaluation, where oral swabs were performed on each of them. The terminal part of the swab was cut and put into 2ml cryovials containing 0.5ml of Dulbecco's Modified Eagle Medium (DMEM) containing 1% of penicillin 10,000,000 U/l. The swabs were frozen and stored at -80°C.
The post-mortem examination was performed according to standard procedures [32]. Macroscopic findings and the collected samples were summarized on a specific worksheet (Annex I, see page 101).
Samples collected from each animal for PCR and histopathology were brain, liver, kidneys, tongue, pharynx, trachea, esophagus, lungs, stomach, gut, heart, genital tract, spleen, and shell. Eyes and gums were included in the sampling list if lesions were macroscopically detected. Two samples of each organ were collected for PCR. Of these, one was stored in an

empty cryovials, the other one in a cryovials with 0.5ml of Dulbecco's medium (MEM) added with 1% penicillin solution.
Samples for PCR were frozen and stored at -80°C. Samples for histopathology were fixed in 10% buffered formalin for ten days and then processed for the paraffin inclusion.

PCR:

Tissues were homogenized using TissueRuptor (Quiagen) and submitted for DNA extraction. DNA was extracted using the same extraction kit (QIAamp DNA FFPE Tissue Kit).
DNA concentration and quality were evaluated with a spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc.).
Two different PCR protocols were applied to the study. The first protocol is termed "Pan-Herpes" and was designed as screening PCR to identify the presence of Herpesvirales DNA in the sample. The “PanHerpes” is a nested-PCR using a preserved sequence of the DNA-polymerase as primer (see chapter 5, material and methods).
“PanHerpes” positive samples were then re-evaluated using a TeHV3 and/or a TeHV2 specific PCR (see chapter 5, material and methods).
The same cycling settings were used for both TeHV3 and TeHV2 specific PCR.
Primes for TeHV3 (see chapter 5, material and methods).

Host pathogen interaction immunological study:

The procedure included three main steps: setup of a bacteriophage library, first immunoblot screening using *Testudo graeca* hyper-immune sera coming from a transmission study, and a second screening based on the FACS evaluation of the selected proteins in transformed eukaryotic cells.
Virus and genomic DNA: The TeHV3 strain US1978/98 [1] was grown on *Terrapene* heart cells (TH-1; ATCC-CCL 50 Sub-line B1; American Type Culture Collection, Rockville, MD, USA) according to an established protocol [2–4]. The TeHV3 genomic DNA was obtained from the same strain similarly to what was previously described [2,4].
Phage Library and DNA sequencing:

Library preparation: the DNA fragments to insert into the bacteriophage were obtained according to the manufacturer’s instructions. Distinct batches of US1978/98 genomic DNA were briefly fragmented by restriction digestion with Sao3AI for different lengths of time. The digested DNA batches were then resolved in a 1% DNA agarose gel. The digested batch, whose TeHV3 genomic DNA fragments ranged between 0.2 to 2 Kb, was selected and the

fragments were ligated into the bacteriophages genome using an auto-assembly bacteriophages kit (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US).

Phage amplification: The library was amplified according to the manufacturer's instructions (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US). Lambda bacteriophages containing TeHV3 genomic fragments were initially briefly expanded, infecting the *E. coli* bacterial strain XL1- MRF (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US). Bacteria were incubated with the bacteriophage suspension for six hours at 37°C while shaking. Following this the suspension contained both bacteria; bacteriophages were mixed with LB-top-agar and overlaid on LB-agar in Petri dishes and incubated overnight at 37°C. The plates showing plaques of lysis were then overlaid with 8ml of SM buffer to harvest the bacteriophages. The buffer was then recovered and stored at +4C. The bacteriophage suspension was subsequently titrated by ten-fold dilutions, using the procedure described above, to determine the optimal concentration of the phage necessary to infect the bacterial cells.

Sera collection: TeHV3 seropositive and seronegative sera samples were obtained from Greek tortoises [*Testudo graeca*] that were experimentally infected with either one of two strains of TeHV3 (US1978/98 and D4295/75) [3] or from spontaneously infected *T. graeca*.

Library screening: Two consecutive screenings were performed on the bacteriophage library. In both XL1-MRF *E.coli* were infected with the selected suspension of bacteriophage as described above and incubated at 37°C over night. The number of clones carrying a fragment of the TeHV3 genome was assessed by white/blue selection carried out according to standard protocol [3]. Plates were then cooled for 1hr at +4°C and then overlaid with a nitrocellulose membrane (0.5 µm, Ø82 mm, PROTRAN BA 85, REF. 10401116, CONV.-NO EW0051-1) that was left in place for 15 min. On each membrane and dish, reference markers were drawn to properly orientate the membrane for the selection of the positive clones. Membranes were then immersed in blocking solution (TBS-T with 5% skimmed milk powder-Brand) for 1hr at room temperature on a rocker. Membranes were then washed twice with blocking solution for 5 min each, and twice with PBS for 30 seconds each. In the first screening the membranes were incubated with hyperimmune tortoise sera coming from the first transmission study performed on TeHV3 by Origgi et al. [3]. During the second screening, membranes were incubated in a mix of tortoise sera, composed of half hyperimmune serum from the transmission study and half from ELISA positive, spontaneously infected tortoises. Sera were diluted 1:20 in blocking solution for 1hr and washed as described above. A biotinylated mouse monoclonal antibody (HL154) was directed against the heavy chain of *Testudo* spp. IgY was used as secondary antibody [2] at a concentration of 1µg/ml in PBS. The membrane was incubated with the primary antibody for 1hr on a rocker at room temperature and then washed as described

above. A mixture of 2 µl AP-Streptavidin (Zymed, 43-4322) and 2 µl of polyclonal antibody rabbit anti-mouse (Jackson ImmunoResearch, 315-005-003) diluted in 1ml of PBS was then applied to the membrane for 1 hour at room temperature. Following five washes of 10 seconds each with distilled water, the membrane was finally stained with the chromogen substrate composed of 45 µl NBT (18.5 mg/ml nitro blue tetrazolium chloride) with 35 µl BCIP (9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate) diluted in 10ml of SM-Buffer (10ml 1M carbonate buffer + 10 ml 100mM MgCl2 + 980ml H2O). The chromogenic reaction was closely monitored for three minutes; then the membrane was rinsed 4-5 times with distilled water and immediately dried with Whatman 3M paper (Sigma-Aldrich, US).

Positive bacteriophages were punched out from the plate using a 20µl tip. These phages were amplified and stored at 4°C according to the manufacturer's instructions (ZAP Express Predigested Vector Kit and ZAP Express Gigapack Cloning Kits, Agilent Technologies).

The positive clones from the primary screening were then expanded and tested again with hyperimmune tortoise sera similarly to what is described above. After this, the selected clones were amplified in *E. coli* strain XLOLR to obtain the phagemids as described by the manufacturer (ZAP Express Predigested Vector Kit and ZAP Express Gigapack Cloning Kits, Agilent Technologies). Phagemids were then digested with SAO3AI to obtain the correspondent TeHV3 genomic inserts and resolved in a 2% agarose gel.

Sequencing: Each of the positive clones was then partially sequenced in order to determine the identity of the genomic insert. A mastermix was used composed of 3µl of 10x PCR Buffer (Qiagen, Silicon Valley, USA), 0.4µl of dMTs, 2µl each of FW and RW Turboprimers (Tri-Link, San Diego, USA), 0.1µl of Taq (Qiagen, Silicon Valley, USA), and 21.5µl of DNA-RAN free water. Samples were then placed in a thermocycler (Thermofisher Inc., US) and amplified for the first time. The amplification program consisted of 3 minutes' denaturation at 94°C, then 35x amplification cycles subdivided in 30 sec. at 94°C denaturation, 30 sec. at 65°C annealing and 2 minutes at 72°C elongation, with a final extension of 10 minutes 72°C to exhaust the polymerase. Each PCR amplicons was submitted for automated Sanger sequencing [ABI Prism 3700, Applied Biosystems] using the Big-dye terminator technology. Briefly, a master-mix containing 2µl of Sequencing Buffer 5x (Thermofisher Inc., US), 0.5µl of either FW or RV Primer (10µM), 2µl of BigDye (Thermofisher Inc., US), 2 µl of DNA and ddH_2O up to 10 µl was prepared. Sequencing was carried out using the same primers used for PCR amplification as described above.

Fluorescence Activated Cells Sorting [FACS] Validation in VERO cells:

Eukaryotic cell transformation: extracted phagemid clones were used to transfect VERO cells. Briefly, 10^5 Vero cells were seeded in each well of a 6-well plate (TPP, Trasadingen, Switzerland) one day prior to transfection. On the following day, one microgram of phagemid was mixed with 135µl of OptiMEM (Thermoscientifc, US) and 3µl of TransIT-LT1 (Mirus Bio LLC, Medison WI, US) in Eppendorf tubes. A single well was used for each of the tested phagemids. Tubes were incubated for 15 minutes at room temperature. The suspension was then added to each well. Plates were gently mixed and incubated overnight at 37°C. Each phagemid was transfected in duplicate.

Cell membrane fixation and permeabilization: one of the duplicates underwent permeabilization, while the other did not. Initially, the cell monolayers of each well were fixed with cold (4°C) 10% buffered formalin directly into the well for 5 minutes. Later, cells were chemically and mechanically detached by using PBS 0.5 mM EDTA incubated for 30 minutes at 37°C and sterile cells scrapers (TPP, Switzerland), respectively. Detached cells were collected in 10 ml Falcon tubes (TPP, Switzerland) and centrifuged at 300g for 15 minutes. After two PBS washes, cells were resuspended in 10ml PBS. The cells that underwent permeabilization were instead resuspended into a 0.2% Tween 20 PBS solution, and incubated at 37°C for 15 minutes. After permeabilization cells were pelleted at 300g for 5 minutes, and finally resuspended in PBS.

Cellular staining: The same staining procedure was performed for both permeabilized and not permeabilized cells to evaluate the expression of intracellular and superficial proteins. Cell tubes were centrifuged at 300g for 15 minutes and resuspended in to 300µl of DPBS with 15µl of seropositive *T. graeca* serum (1/20). Tubes were incubated at 4C° for one and a half hours. After incubation, cells were centrifuged (300g x 15min), washed two times with DPBS, and then resuspended in 1ml DPBS with 0.1µl of primary antibody (HL1546 mouse anti turtle-IgY), and incubated for 1 hour at room temperature. Primary antibody was washed away as described above. Later, a solution of 1ml DPBS with 0.5µl of secondary antibody (rabbit anti mouse, Jackson ImmunoResearch, 315-005-003) were incubated for 1 hour at 4°C with the cells. Following an additional wash, cells were resuspended in a solution of 1ml DPBS with 1µl of a green fluorochrome anti rabbit (Alexia fluo 488, Thermofisher, US). Cells were incubated for ninety minutes and then centrifuged (300g x 15 min), washed three times with DPBS, and resuspended in PBS.

Phagemid editing and bacterial cell transformation:

Phagemid editing:

Different techniques have been used to "remove" TeHV3 genomic DNA fragments one at the time from the original phagemids, including:

1) Enzyme restrictions: different sets of enzymes were used to cut the DNA fragments based on the phagemid sequence. Only single cutter restriction enzymes were used, to avoid multiple cutting of the genomic sequence.
2) PCR direct site mutagenesis

To increase the probability of successful transformation, enzymatic restriction products were purified and inserted in a new, smaller cloning vector using ligase reaction. The cloning vector used is called pET30C+ and is a high copy number plasmid.

Bacterial cell transformation:
Transformation was performed according to standard procedures, samples were plated on LB agar with added antibiotic solution.

Results:

Selection of new prospective cases:

Among all the twenty-eight selected cases suspected of infection, only one showed histological changes similar to those induced by the TeHV3, and specifically heterophilic glossitis and rhinitis. None of the selected samples were PCR positive and no viral particles were isolated from the frozen tissues.

Genome:

The complete genome of TeHV3 was encoded, although two genome areas characterized by the presence of numerous tandem repeats were difficult to complete and the exact sequence was uncertain.

Host pathogen interaction study:

Bacteriophage library screening and clones sequencing:
The optimal working dilution for the bacteriophage suspension was determined to be 10^{-3}.
The initial screening of the phage library was carried out with hyperimmune sera obtained from experimentally infected Greek tortoises [5] revealed 16 positive out of approximately 5,000 clones, accounting for 0.32%.

A second screening carried out on each of the amplified clones confirmed the positivity of clones 1 and 6 (12.5%), whereas all the other clones were negative and were detected as false positive clone.

Clone 1 contained a 1.8 Kb long DNA fragment corresponding to the entire gene of the ribonucleotide reductase and to a partial sequence of the major capsid protein encoding gene of TeHV3. Clone 6 contained a 2.3 Kb long DNA fragment corresponding to the partial sequence of the glycoprotein B encoding gene.

The initially positive 16 clones were screened a second time by using a mix of both experimentally and spontaneously infected Greek tortoise sera. Among the 16 tortoises 6 showed a positive result (37.5%), but only 5 carried DNA inserts (Figure 2).

Clone 1a contained 2 Kb of DNA containing part of the large sub-unit of ribonucleotide reductase and a fragment of the tegument protein genes. Clone 1b contained 2 Kb of DNA encoding for a partial sequence of the glycoprotein B gene. Clone 3 contained 500 bp of DNA encoding for part of the envelope protein gene. Clone 6 contained 300 bp of DNA encoding for a fragment of the immediate early 2 gene. Clone 7 contained 600 bp of DNA encoding for a part of the uracil DNA glycosylase gene.

Eukaryotic cells transformation and FACS:

The clones that were confirmed positive during the second screening were then selected for expression in Vero cells. Expression of the partial sequences of the genes carried by the positive phagemids was assessed by FACS analysis. We considered that in order to validate the selected clones, it was necessary to test them in a different setting from that used initially to provide an independent second line of evidence to our results.

New insight:

Singer sequencing of the phagemid demonstrated that the gB gene was actually antisense to the phagemid ORI. Two genes were detected to be in agreement with the phagemid origin of replication and were extracted or deleted from the phagemid as described in the materials and methods. The extracted samples were inserted in new cloning vectors (pET30C^{+}). Both modified phagemids and new generated plasmids were used to transform E. coli cells. However, despite all efforts, no viable colonies were obtained.

Discussion:

This work aimed to start evaluating the host-pathogen interaction between *Testudo graeca* and TeHV3 by investigating the most relevant immunogenic proteins of this virus.

The viral proteins were expressed in bacteria using a bacteriophage display library, and serologically screened using hyperimmune *Testudo graeca* sera obtained from a transmission study [5]. Phage library is a molecular technique widely used for investigating infectious diseases in human beings [6] and occasionally in veterinary medicine [7,8]. Phage displays have been used with pathogens of different mammals [7], birds [8] and fish [9] species, but this was the very first time they were applied to reptiles.

Among the herpesviruses, the most antigenic proteins identified both in human beings and animals [10–12] are the major tegument protein (UL37) and the family of glycoproteins [13].

Glycoproteins are a class of capsidic proteins involved in the attachment and entry of viral particles into the host cell [14].

Three major classes of glycoproteins have been identified (B, D and G) and all of them seem to have a crucial role in the infection of the host cell by the virus [15–17].

Among the glycoproteins, the gB is considered a relevant and highly conserved immunogenic molecule in herpesvirales [18,19].

During this experiment six proteins were serologically detected as immunorelevant, but only the gB was positive to the FACS.

The expression of the selected clones in a eukaryotic system implies the glycosylation of the residues to the candidate immunogenic peptides, differently from what occurs in prokaryotic systems. Glycosylation was expected to closely reproduce the native state of the viral peptide, providing more informative data concerning the actual antigenicity of the candidate peptides. Transfected cells were tested either with or without cell membrane permeabilization. Unambiguous positivity was confirmed by FACS analysis only for the surface expression of the clone 1b, encoding for the partial sequence of the gB gene. No other clones showed convincing positive signals, despite cell permeabilization, increase of amount of transfected DNA phagemids, prolonged expression time of the transfected cells, or the use of other fluorescent dyes.

The expression of the proteins in eukaryotic cells may have led to conformation changes not allowing allosteric binding of the antibody to the protein, with consequent misdetection. However, the possibility of false positives has to be considered and the proteins should be retested using a different technique.

This is the very first study evaluating the immunological response of reptiles to viruses and the host-pathogen interaction. As previously demonstrated in other herpesvirus, the gB represent an immunologically important antigen, based on which new diagnostic tools and vaccinations can be developed.

Unfortunately, deeper investigation demonstrated the gB sequence was antisense to the plasmid ORI, and thus gB cannot be responsible for the immunogenic response we detected in the previous experiment.

We cannot exclude the immunogenic role of gB in TeHV3's response, but further investigations are needed to clarify this point. Despite all the attempts we made to transform bacterial cells with the new constructs we prepared, no viable colonies were obtained, which might correlate with different problems including:

- Unsuccessful transformation protocol
- Genomic toxicity induced by the genes we edited

Due to the multiple unsuccessful attempts, a biotechnician was requested to perform bacterial transformation but again without success.

We are currently still working on the problem.

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Chapter 1: History of reptile medicine.

Herpetoculture (Herpeto= reptile; culture= breeding) is the activity of keeping and breeding live reptiles in captivity for both conservational, recreational or commercial purposes.

Humans and reptile interaction has its roots at the beginning of human history. At the dawn of history, cohabitation with dangerous (e.g. crocodiles, caimans, and alligators) or easy to catch (e.g. turtles and tortoises) reptiles pushed people to start hunting these animals for both defense and food. Different cultures include reptile meat as traditional food, for example, South Africa (crocodiles) or China (turtles and tortoises). As the years went by, human hunting abilities and human population density increased exponentially, leading to the extinction or near extinction of different animal species, including numerous reptiles. One of the most relevant examples is the hunting of loggerhead (*Caretta caretta*) and olive riddle (*Lepidochelys olivacea*) sea turtles during the "Golden age of piracy" (1650-1730). Because of the inability to preserve food during ocean crossings, pirates used to hunt sea tortoises for meat, but this habit almost caused the extinction of the above-mentioned species. In addition, reptile meat has been extensively used in Asian traditional medicine, for example in China. Commercial (farming), conservational, and recreational breeding of reptiles had separate historical timelines.

The first attempts of herpetoculture were made to farm reptiles for food and traditional medicine. One of first reptile herpetoculture centers was a Japanese (Fukagawa, Tokyo) tortoise breeding center, which dates back to 1866.

Reptile breeding for conservational purposes only started later. One of the most relevant examples is the St. Augustine Alligator Farm Zoological Park, Florida (1893).

Over time the distinction between farming and conservational purposes blurred, and it is now more common to find farms that release animals into the wild, and breeding centers that cull animals to sell meat and leather. The reason for this is that at least in some areas of the world, there is less demand for reptile meat and leather, also because of people's increased awareness of animal welfare and extinction threat of some reptile species. The reduced capture of wild animals and the release of reptiles from farms frequently result in breeding centers being overpopulated, forcing them to cull some of their stock.

Herpetoculture of pet reptiles has a more recent history. First attempts can be dated back to the 1950s, but the real success of pet reptiles started in the 1970s.

Because of the animals' complex maintenance needs and the limited knowledge about their medical conditions and pathology, numerous diseases began to affect pet reptiles' health. For this reason, standard veterinarian knowledge was not enough to take care of reptiles, and vets specialized in exotic pets started to become popular.

From that time until now, giant steps have been made in reptile medicine, which can now be compared to domestic pet medicine.

Chapter 2: Reptile trading.

Reptile trade represents a relevant business in both the USA and Europe. According to Auleiya et al. (2016), [1] the import of live reptiles in Europe for the year 2015 was worth 7 million dollars, during the same year the import of reptile leather was worth 100 million dollars. In 2013 USA legally imported reptiles worth 20 billion dollars; in the same year the illegal trade in reptiles seems to account for 232 million dollars.

Although reptiles represent an economically important trade in the US and various EU countries, the exchange of allochthonous species accounts for regulatory, biodiversity and health problems.

To regulate the trade in exotic species, the US joined the International Union for Conservation of Nature (IUNC) in 1948. To protect the endangered animal and plant species, the IUNC established a "Red List", which contains all threatened animals and plant species. The IUNC list includes about 45% of the known reptile species, of these 180 are classified as critically endangered, 361 as endangered, and 403 as vulnerable. Despite the established measures, the remaining 55% of non-classified reptile species are not subject to trade restriction and are progressively threatened by the international pet and leather trade. Furthermore, smugglers use strategies to export protected animals that include the use of false captive breeding certificates. [1, 2]

Within the period 2004–2014, the EU member states officially reported the import of 20,788,747 live reptiles (CITES and non-CITES species) (Eurostat, 2015). With 6,101,040 live specimens, Germany was by far the largest importer within the EU, followed by Great Britain (US$3,469,109), Spain (US$2,912,171), the Czech Republic (US$1,899,420), and Italy (US$1,780,546).

The introduction of all these allochthonous species, which share the same ecological niches as the autochthonous species and may carry exotic diseases, represents a huge risk for local species. Among reptiles, this is well represented by the red-eared slider turtle (*Trachemys scripta*) which is characterized by a very high fertility, progressively replacing the allochthonous freshwater turtles (*Emydid* spp.) all over the EU.

Furthermore, the introduction of allochthonous reptiles represents a risk to human health. One of the most relevant pathogens related with reptile import/export is *Salmonella* spp. [3] Cases of reptile mediated salmonellosis have been demonstrated both in the US and the EU, especially in children. In 2017, the CDC (Center for Disease Control and Prevention) reported a multistate outbreak of *Salmonella agbeni* in the USA, related to the contact with pet turtles.

Reptile trade in Italy:

Although few official data are available about the reptile trade in Italy, this country represents one of the most relevant European nations involved in exotic and non-exotic reptile trade. Most recent data available about the current reptile trade in Italy are from 2015, and range from about US$1.5 million (Erostat, 2015) to US$1.8 million (Assalco-Zoomark 2015). [1, 2] The most commonly traded reptiles in Italy are tortoises, turtles and snakes, followed by lizards. These data are more representative than real, as a matter of fact the institution of mandatory microchipping and birth certificates for animals included in the CITES list introduced in 2011, which includes many species from the IUNC "Red list", made many previously legally owned animals illegal. Furthermore, the offspring from these animals are frequently sold on the black market at lower prices, representing a true underground economy.

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Chapter 3: Reptiles' immune response to viruses.

The immune response against microorganisms such as viruses is a complex mechanism, which involves both innate and acquired immunity.

The main difference between the two systems is that innate immunity is capable of producing a stereotyped response against non-self, using highly conserved recognitions patterns, without a previous sensitization of the host. In contrast, acquired immunity is not designed to activate on first contact, but is needed to produce very specific and highly effective molecules, specifically designed against that pathogen.

Among innate immunity trigger systems, the germline-encoded pathogen pattern recognition receptors (PRRs) are the most relevant. Pattern recognition receptors includes C-type lectin receptor (CLRs), Toll-like receptors (TLRs), Intracellular retinoic acid inducible gene – I (RIG-I)-like receptors (RLRs), nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs), and the Pyrin-HIN domain (PYHIN) receptors. Among the receptors mentioned above, each group comprises different members that are responsible for recognizing a specific pathogen pattern.

In human medicine, the role of TLRs and RLRs is well established, while the role of NRLs is not completely clear. Activation of TLRs and RLRs is associated with the release of the Interferon type I (INF-I), which is responsible for upregulating the genes inhibiting viral replication and is involved in adaptive immune system activation. Furthermore, a new type of INF has been identified as antiviral molecule of the INF type III. The latter has a little structural homology with INF-I and activates different heterodimeric receptors.

The release of INF-I and III induces the activation of innate immunity cells like natural killer and dendritic cells, which are responsible for destroying the source of the stimulus and contributing to antigen processing and adaptive immune system activation. While natural killer cells only destroy the host cells to try to prevent pathogen replication, dendritic cells are involved in antigen presentation (included in the so-called group of "antigen presenting cells"). Once dendritic cells have phagocytized, the pathogen starts to elaborate the most relevant antigens which are then exposed on the cellular surface by a specific molecule named the Major Histocompatibility Complex (MHC).

Another relatively new mechanism involved in host immune regulation is autophagy (auto= self and phagy= eating). Autophagy is the mechanism that cells use to adapt to specific metabolic requirements or reduce energy consumption by reducing or increasing the number of specific organelles in the cytosol. Autophagy regulation in mammals has been demonstrated

to be influenced by some viruses that force cells to regulate their metabolic activity accordingly to viral requirements. Recently (2009) [1], autophagy has been demonstrated to be involved in the process of antigen presentation via MHC I and II.

Very little information is available about the role of reptiles' immune system in preventing and controlling viral infections.

In reptiles, the presence of TLRs has been hypothesized based on genome wide sequences aligned with birds, fishes, and mammals. [2-6] There seem to be significant differences in TLRs total number among both reptile classes and species, but TLRs 2 and 4 are the most represented among reptiles. [2-6]

An interferon-like molecule with antiviral activity has been described in reptiles [7, 8] and its release has been documented in *Testudo graeca* kidney primary cultures infected with West Nile virus, Semliki Forest virus, Newcastle disease virus, and Sendai virus. A similar factor was later documented later in *Terrapene* heart cell cultures [9] infected with Louis encephalitis virus. The chemical structure of these INF-like molecules is similar to the one of mammals, but differ for some amino acid sequences.

Reptiles possess both phagocyte cells and lymphocytes. In contrast to mammals, professional phagocytic cells in reptiles not only include macrophages and dendritic cells, but also B-lymphocytes. The phagocytic activity of reptiles' dendritic cells has been documented in splenic macrophages of Indian leaf-toed gecko. [10] The ability of reptiles' macrophages to phagocytize microorganisms seems to correlate with both daily and seasonal circadian rhythms, and peaks in correspondence with the annual reproductive cycle have been observed. [11, 12] Although post phagocytosis mechanisms have been investigated in dendritic cells after bacterial phagocytosis [13] and they seem similar to those of mammals, there are no studies on viral antigen processing in reptiles.

Antigen presenting cells express antigen on their cellular surface thanks to MHC, which is also described in reptiles. As in mammals, two MHCs have been described in reptiles.

Natural killer lymphocytes (or functionally similar cells) have been described in different reptile species, including turtles, [14-18] but no information is available about the possible role of these cells in antiviral response.

Autophagy is a highly conserved process in cell biology, although no information is available about the presence of this regulatory mechanism in reptiles.

Mechanisms similar to those described in mammals are present in other vertebrate classes including fishes and birds, suggesting the presence of an evolutionary continuum among vertebrates. [19, 20]

The limited knowledge of reptile immune systems makes it difficult to understand host-pathogen interaction in these animals, and consequently reduces the therapeutic margin available in these species.

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Scientific publication related to the research argument:
Francesco C. Origgi, Marco Tecilla. Chapter 2: Immunology of reptiles in: Infectious disease and pathology of reptiles, 2ed. Vol.1 (to be published).

Chapter 4: Herpesviruses of chelonians.

The following content will be published in the Italian book: Medicina e chirurgia delle tartarughe e delle testuggini (Medicine and surgery of turtles and tortoises)
Chapter name: Patologie infettive dei cheloni (Infectious diseases in Chelonians)
Authors: Marco Tecilla, Francesco C. Origgi.

Introduction:

Herpesviruses are DNA viruses with envelopes, belonging to the order *Herpesvirales*, family *Herpesviridae*. Herpesviruses that infect turtles are considered part of the *Alphaherpesvirinae* subfamily, but in most cases this is a hypothetical classification based only on the nature (necrotizing) of lesions induced by these viruses. Only the Chelonian herpesvirus 5 and the *Testudinid herpesvirus* 3 have been demonstrated to genetically be part of *Alfaherpesvirus*. [1-3] The classification of herpesviruses in turtles is complex and not yet entirely clear. Among the Chelonians' herpesvirus only the *Chelonian herpesvirus* 5 is included in a new and specific genus called *Scutavirus*, which currently contains only this virus. [2] Herpesviruses of turtles are distributed as follows:

1. Herpesvirus of marine turtles: *Chelonid herpesviruses* (ChHVs: 1,5,6, Loggerhead genital-respiratory herpesviruses, Loggerhead orocutaneous herpesvirus).
2. Herpesvirus of freshwater turtles: *Chelonid herpesvirus* 2 and 3; *Emydid herpesvirus* type 1 and 2; *Glyptemys herpesvirus* type 1 and 2, *Terrapene herpesvirus* 1.
3. Herpesvirus of tortoises: *Testudinid herpesvirus* [TeHV-genotype 1 (TeHV-1), 2 (TeHV-2), 3 (TeHV-3), 4 (TeHV-4)].

For the sake of readability, individual species of herpesviruses that infect turtles will be addressed individually.

Herpesvirus of chelonians:

1 Chelonid herpevirus (ChHVs):

ChHVs herpesviruses are the most commonly isolated and among the best characterized herpesviruses in turtles. [4] These viruses have been reported in different species of aquatic turtles, including *Clemmys marmorata*, *Chrysemys picta*, *Graptemys* spp., and *Chelonia Mydas*. [4] Within the *Chelonian herpesviruses*, different viruses are identified:

1.2 Chelonian herpesvirus 1,2,3,4,5,6.

1.2.1 Chelonid herpesvirus 1(ChHV-1) *or gray plaque disease*

Description/Overview: this was the first herpesvirus to be described and was associated with the onset of gray plaque (Gray Patch Disease) on the skin of green turtles (*Chelonia mydas*). The disease was first described in a population of green turtles located in the west of India, and subsequently recognized in other subpopulations of *Chelonia mydas* located all over the world. [5, 6]

Zoonotic potential: not reported.

Clinical presentation: The virus has only been reported in captive bred green turtles (*Chelonia mydas*), with clinical signs onset ranging between 56 days and one year of age. [7]

Clinical presentation: the pathology is associated with the appearance of cutaneous circular papules, which often ulcerate. Papules usually regress spontaneously in a generally short time, although variability has been observed. In animals with severe symptoms, mortality varies between 5% and 20%. [7]

Risk factors: animals bred in captivity. It is believed that a high density of animals favors the spread of the virus. [7]

Pathology: this disease is associated with the appearance of areas of hyperkeratosis on the head, neck and flippers. Hyperkeratotic areas frequently undergo focal necrosis of the spinous and granular layers, leading to the formation of papules. Histologically, the lesions are characterized by the presence of intranuclear amphophilic inclusion bodies in epidermal keratinocytes. [6, 7]

Transmission: the disease has been experimentally transmitted through skin scraping but the route of transmission in nature is not known.

Distribution of the virus: between the end of the 70s and the beginning of the 90s the virus was isolated in different populations of captive bred *Chelonia mydas* in Asia. Subsequently, the pathology was no longer observed until relatively recently. Today it is believed that the disease has a world-wide distribution.

Treatment: there is no pharmacological treatment to contain the effects of the virus. International guidelines suggest to quarantine infected animals for a minimum of three months, reduce the herd stress and reduce the concentration of animals in the tub. It has been observed that lesion severity and incubation time are influenced by tank water temperature. The optimal water temperature to prevent or reduce the onset of viral related lesions is 25°C; it is advisable to use this parameter of reference both for prophylactic and curative purposes. [8] If the cutaneous lesions undergo ulceration, the administration of a prophylactic antibiotic for the prevention of secondary infections is advisable.

Differential diagnosis: lesions induced by this virus are very peculiar, however a differential diagnosis should include a possible dermatophytoses and the ulcerative septicemic skin disease (SCUD).

1.2.2 Chelonian herpesvirus 2 (ChHV-2)

Description/Overview: this was identified for the first time in two western pond turtles (*Clemmys marmorata*) and subsequently in the false map turtle (*Graptemys pseudogeographica*).

Signaling: The virus was reported in the western pond turtle (*Clemmys marmorata*) and subsequently in the false map turtle (*Graptemys pseudogeographica*).

Clinical presentation: the pathology is associated with the appearance of lethargy, anorexia and subcutaneous acute edema (approximately one week). [9, 10]

Risk factors: in one of the two reports of the disease, the animals were kept with others aquatic turtles (*Graptemys* spp.). Herpesvirus associated lesions detected in *Graptemys* spp., including subcutaneous edema, pulmonary edema and necrotizing hepatitis were subsequently reported in other aquatic turtles of the genera *Clemmys marmorata* and *Chrysemys picta* which could therefore have had the function of reservoirs. [10] The lesions in the turtles mentioned above are very similar to those found subsequently in other turtles of the group *Emydidiae* spp., in which *Emydid herpesvirus* 1 was identified; a direct correlation, however, between these viruses has never been investigated. [11]

Pathology: macroscopically the subjects are characterized by the presence of diffuse subcutaneous edema, pulmonary edema and a fatal necrotizing hepatitis. Upon microscopic examination, the classic intranuclear, amphophilic inclusion bodies from herpesvirus were identified in the liver, kidney and pancreas. [10]

Zoonotic potential: not reported.

Transmission: unknown.

Distribution of the virus: all the above-mentioned reports are from North America, but the true distribution is not known.

Treatment: there is no pharmacological treatment to contain the effects of the virus. The trend of the acute disease with severe hepatic dysfunction does not leave much therapeutic margin. It is suggested to isolate animals immediately upon the onset of symptoms.

Differential diagnosis: the presence of diffuse subcutaneous edema is typical enough, however septicemic forms with severe hepatic impairment and ranavirus infections should be taken into account.

1.2.3 Chelonian herpesvirus 3 (ChHV-3)

Description/Overview: it was described for the first time in 1980, in a *Chrysemys scripta*. [9]

Clinical presentation: the virus has only been identified in the American pond turtle (T*rachemys* [*Chrysemys*] *scripta*).

Clinical presentation: the only report is from one turtle previously treated for the presence of an aural abscess that suddenly died within after a week after the end of the antibiotic therapy.

Risk factors: not known.

Pathology: macroscopically the subject was characterized by the presence of pulmonary edema, friable liver with a brownish-green discoloration, and the gall bladder was severely extended by the presence of bile. The histological examination highlighted numerous foci of coagulative necrosis in the liver, associated with a minimal inflammatory response and the presence of intranuclear amphophilic inclusion bodies. In both the lungs a fibrinous bronchopneumonia, associated with the presence of micro-hemorrhage and numerous desquamate epithelial cells, often containing bodies similar to those of the liver were observed. [9]

Zoonotic potential: not reported.

Transmission: unknown.

Distribution of the virus: at the moment it is only reported in North America.

Treatment: there is no pharmacological treatment to contain the effects of the virus. The trend of the acute disease with severe hepatic dysfunction does not leave much therapeutic margin.

Differential diagnosis: the symptomatology is unspecific enough; the presence of pulmonary edema and liver degeneration with acute trend includes forms of septicemic bacterial, protozoal (e.g. amoebiasis) and viral diseases (e.g. Iridovirus).

1.2.4 Chelonian herpesvirus 4 (ChHV-4)

Description/Overview: this virus was described for the first time in 1985, in a mixed consignment composed of Argentine turtles (*Geochelone chilensis*) and red-footed tortoises (*Geochelone carbonaria*). However, only the Argentine tortoises showed clinical signs of infection.

Clinical presentation: the virus was identified in Argentine turtles.

Clinical presentation: the classic signs related to the infection are the presence of necrotizing stomatitis and glossitis, which often becomes purulent for the onset of secondary bacterial infections (mouth rot).

Risk Factors: unknown.

Pathology: macroscopically necrotizing glossitis and stomatitis that can extend to involve the retropharyngeal tissues, the hard palate and nasal cavities tissues are present. Serous atrophy of the liver is often present. Histologically, the tissues of the oral and the nasal cavities are extensively necrotic and ulcerated, and pseudo membranes are present. The intranuclear amphophilic, inclusion bodies are generally numerous and mainly identifiable in the degenerated and necrotic cells. Often mixed morphology bacteria (cocci, bacilli, and coccobacilli) of secondary eruption are detected.

Zoonotic potential: not reported.

Transmission: not known.

Distribution of the virus: at the moment it is only reported in North America.

Treatment: not known.

Differential diagnosis: the symptomatology is specific enough; a tentative diagnosis can be issued for the presence of stomatitis and glossitis in an Argentine turtle.

1.2.5 Chelonid herpesvirus 5 (ChHV-5) *or Cheloninan fibropapilloma-associated herpesvirus* (CFPHV) *or Fibropapillomatosis* (FP)

Description/Overview: this virus is associated with the occurrence of cutaneous fibropapillomas and visceral fibromas in marine turtles. This is a virus with global distribution and is able to infect various marine turtles. [4] Recent molecular investigations have suggested that the virus can be maintained in the environment by the presence of healthy carriers, in which concentration of the virus in the skin seem higher compared to healthy turtles. [12] Although ChHV5 has actually been classified as *Alfaherpesvirus*, is not yet known with certainty if it goes latent within the trigeminal nerve ganglion or in other in structures. The viral genome sequencing highlighted that the ChHV5 genome contains some genes typically identified in gamma- and beta-herpesviruses' genes; this feature allowed to classify this virus in a new genus, the *Scutavirus*. However, the oncogenic mechanism behind the onset of tumors is not yet known. [2]

Signaling: the virus is able to infect marine turtles of the species: *Caretta caretta*, *Eretmochelys imbricata*, and *Lepidochelys olivacea*. [4]

Clinical presentation: the virus is associated with the formation of pedunculated or sessile tumors, with a smooth or rough surface at skin and visceral level. The cutaneous neoplasms are associated with problems in capturing food, in movement and in vision. The visceral tumors are associated with the obstruction and compression of hollow organs and, less frequently, of parenchymatous organs.

Risk factors: are not known. It is believed that, given the behavioral habits of these animals, a critical moment for the transmission is the coupling. Anecdotally, leeches were deemed possible carriers and vectors of the disease but there is no scientific evidence in this respect.

Pathology: it is believed that the virus gives rise to a viremia as soon as it enters the body, and that it subsequently replicates in the skin (including in the slab and the carapace) and mucosa (especially conjunctiva). The replication of the virus gives rise to the formation of sessile tumors, with a central axis of connective tissue, whether or not associated with the presence of irregular hyperplasia of the epidermis, identified as fibropapillomas. When the disease progresses temporally, many fibropapillomas transform into tumors with exophytic but not sessile growth, originating from the connective tissue; these tumors are characterized by a central core composed of well differentiated fibroblasts and collagen coated by epidermis that are regularly or irregularly hyperplastic, called fibromas. Subsequently to the appearance of skin fibromas, in the terminal phases of the disease, viral replication gives rise to the formation of fibromas even at visceral level. Occasionally, the collagen matrix of the visceral fibropapillomas may have a gelatinous (mixoid) appearance and for this reason have also been identified as mixofibromas. More rarely, connective tissue tumors were also identified with criteria of cellular atypia suggestive of low grade fibrosarcomas. Although they have been reported at the level of different organs, fibrosarcomas would seem to be the most common at the level of the heart and in particular of the right atrium. [13-15]

Zoonotic potential: not reported.

Transmission: the route of transmission of the virus seems to be horizontal.

Distribution of the virus: the virus has a global distribution. [16]

Treatment: there is no treatment. Surgical resolution attempts have been reported in literature attempts to treat skin tumors in subjects that have few lesions, however the tumors tend to recur.

Differential diagnosis: lesions are typical of this disease.

1.2.6 Chelonid herpesvirus 6 (ChHV-6) *or Lung, Eye, Trachea disease* (LETD)

Description/Overview: this is a pathology described only in marine turtles, characterized by the appearance of lesions of the ocular globes, oropharynx and lungs. The pathology was described for the first time in a herd of turtles of the Cayman Islands, where it occurred as an epizootic disease in animals aged between 1 and 2 years. [17] Unlike other viruses that infect the turtles, this was isolated and cultured in tissue cultures, allowing to develop molecular methods for its identification. [4, 18] In literature the acronyms LETD is used to indicate the disease while LETV is used as a synonym for ChHV-6.

Signaling: the virus is able to infect marine turtles of the species *Chelonia mydas* and would seem to have a higher tropism toward subjects aged between 1 and 2 years. Typically, the pathology occurs between January and August. [7] The presence of antibodies against this virus has also been demonstrated in turtles of the species *Caretta caretta*, in which, however, symptomatic forms of the disease have never been reported. [17]

Clinical presentation: infection is associated with the formation of skin ulcers, linked with the accumulation of mucus at the level of the eyes and oropharynx. During the acute phase of the disease animals have dyspnea, difficulties in staying afloat, keratitis, conjunctivitis, tracheitis and pneumonia, that persist for 2-3 weeks. Subsequently the symptomatology loses intensity. However, infected subjects usually die within some weeks to several months after the disappearance of the signs, probably because of the respiratory functional damage caused by the virus. [7, 18] Secondary eruption gram-negative associated bacterial diseases have been demonstrated in an incidence study over a ten-year period. [7] The mortality associated with this disease varies from 8% to 38% and rises to 70% in the case of secondary infections. It is believed that the virus has an incubation period of 2-3 weeks.

Risk factors: are not known, however the presence of this disease in mainly farmed and aquarium animals suggests that overcrowding and poor hygienic conditions may play a fundamental role in the onset of clinical symptoms.

Molecular diagnostics: the virus can be identified through PCR, ELISA on plasma, Western blot and immunohistochemistry.

Pathology: at external examination, the presence of a grayish exudate with a mucoid aspect at the level of the eyes and mouth is the most typical finding. The presence of skin ulcers is relatively constant. At the opening of the oral cavity there is edema of the glottis and pharynx with mucus, and sometimes with necrotic material. Hepatomegaly can be observed, but the main feature is the presence of multifocal whitish foci, which in cross-section are also present within the parenchyma (foci of necrosis). The lungs are often hyperemic and decreased in consistency; on cut sections an abundant grayish to yellow exudate is often present. Histologically, the presence of necrosis associated with a mixed inflammatory infiltrate affecting pharyngeal tissues, liver, trachea and lungs is often detected. At the pulmonary level there is severe bronchopneumonia, which can often be associated with secondary bacterial infection.

Zoonotic potential: not reported.

Transmission: the mechanism of transmission is not clear, but it is supposed to occur by direct contact. It has been demonstrated that the LETV remains contagious in salt water for five days.

Distribution of the virus: the virus has a global distribution. [16]

Treatment: there is no pharmacological treatment suitable to contain the viral infection, however the use of antibiotics is recommended to prevent the complications caused by secondary bacterial infections. In the context of the introduction of new animals to preexisting collections, it is recommended that a quarantine of at least three months is performed, both preceded and followed by complete physical examination.

Differential diagnosis: lesions are typical of this disease.

1.3 Genito-respiratory syndrome of the Caretta caretta (Loggerhead genital-respiratory herpesvirus - LGRV)

Description/Overview: this is a relatively little investigated pathology. Although the disease has been described previously, an effective characterization of the etiologic agent was only made in 2007. [19] All cases reported to date have been identified in wild animals, especially from Florida. [19] The disease is characterized by the presence of ulcers affecting the trachea (identifiable endoscopically), cloaca and penis. Outbake reports in literature usually involves single individuals, with the only exception of a *Caretta caretta* die-off of no better specified cause, in which tortoise were LGRV positive. [19] It is not clear if the cause of the death of affected animals is actually the virus, or whether it is a combination of factors.

Signaling: the virus has been isolated only in marine turtles of the species *Caretta caretta*, both male and female. [7]

Risk factors: are not known.

Molecular diagnostics: the virus can be identified through PCR.

Pathology: at external examination, a grayish exudate of a mucoid aspect at the level of the eyes and mouth is noted. Skin ulcers are not always present. At the opening of the oral cavity there is edema of the glottis and pharynx with mucus, and sometimes with necrotic material. The liver can be increased in volume, but the main characteristic is the presence of multifocal whitish foci, which in cross-section are also present within the parenchyma (foci of necrosis). The lungs are often hyperemic and decreased in consistency; cross sections often present abundant grayish yellow exudate. Histologically, the disease is characterized by the presence of hyperplasia of the epidermis, hyperkeratosis, heterophilic inflammation, edema and ballooning degeneration. The inclusion bodies are observable in the epithelial cells of the skin lesions in oral ulcers and in the epithelial cells of the respiratory tract. Most of the lesions induced by this virus are shared with the ChHV6, and it has been proposed that these two viruses are actually one, inducing distinctive lesion patterns in different marine turtle species.

Zoonotic potential: not reported.

Transmission: the mechanism of transmission is not clear, but it is supposed to occur by direct contact. Leeches could be involved in the transmission of the disease.

Distribution of viruses: The virus has a global distribution.

Treatment: there is no pharmacological treatment suitable to contain the viral infection, however the use of antibiotics is recommended to prevent the complications of a secondary burst, caused by bacteria. In the context of the introduction of new animals to preexisting collections, it is recommended that a quarantine of at least three months is performed, both preceded and followed by complete physical examination.

Differential diagnosis: the main differential diagnosis is the ChHV6.

1.4 Loggerhead orocutaneous syndrome (orocutaneous herpesvirus - LOHV)

Description/Overview: the LOH syndrome of *Caretta caretta* is a poorly investigated pathology that is characterized by the onset of ulcers at the level of the oral cavity, pharynx and skin, associated with hyperkeratotic plaques on the tongue and the presence of abundant mucoid exudate in the oral cavity. The virus has also been associated with the occurrence of pneumonia. The virus has only been identified in populations of *Caretta caretta*.

Signaling: The virus has only been isolated in marine turtles of the *Caretta caretta* species. [19]

Risk factors: are not known.

Molecular diagnostics: The virus can be identified through PCR.

Pathology: macroscopically the presence of ulcers at skin level, of the oral cavity and pharynx is observed. On the tongue hyperkeratotic plaques are often present. In the oral cavity abundant mucoid material is often present. Pneumonia is a common finding. Histologically, the epidermis and the mucosa ulceration have ballooning degeneration and intranuclear inclusion bodies, associated with hyperkeratosis and hyperplasia of the epidermal areas nearby. Hetero-

philic inflammation is commonly detected, especially at the margins of the lesions. At the pulmonary level, hyperemia and heterophilic inflammation are frequently observed, occasionally complicated by the presence of secondary bacteria.

Zoonotic potential: not reported.

Transmission: the mechanism of transmission is not clear, but it is supposed to occur by direct contact. Leeches are considered to be a potential vector for herpesviruses of sea turtles.

Distribution of viruses: The virus has a global distribution.

Treatment: there is no treatment. The use of antibiotics is recommended to contain any secondary bacterial infections. It is always advisable to quarantine new animals for at least three months before introducing them, and to isolate infected individuals as soon as compatible clinical signs are observed.

Differential diagnosis: lung, eye, and trachea syndromes must be taken into consideration for a potential differential diagnosis.

2 Emydid herpevirus (EmyHVs)*:*

This group contains two herpesviruses that infect turtles of family Emydidae spp. Emydidis includes two subfamilies and ten genres:

Family: *Emydidae*

Subfamily: *Emydinae*

generes: *Clemmys* spp., *Emys* spp., *Glyptemys* spp., *Terrapene* spp.

Subfamily: *Deirochelyinae*

generes: *Chysemys* spp., *Deirochelys* spp., *Graptemys* spp., *Malaclemys* spp., *Pseudomys* spp., *Trachemys* spp.

2.1 Emydid herpesvirus 1 (EmyHV-1)

Description/Overview: the first case was reported in 2012 in a private collection. A northern map turtle (*Graptemys geographica*) just purchased and held in a quarantine tank, began to

show weakness and nasal drainage; upon radiographic examination multifocal radiopaque areas were highlighted at the pulmonary level. Despite the antibiotic support (cefazidime) the subject died. [11]

Signaling: the disease seems to occur only clinically in *Graptemys geographica*, however four asymptomatic *Chrysemys picta* were PCR positive.

Risk factors: are not known.

Molecular diagnostics: the virus can be identified through PCR.

Pathology: microscopically, the lungs are characterized by hyperemia and edema. The liver is pale in color and moderately enlarged. Histologically, the lungs are characterized by the presence of necrosis of the epithelial cells, inside of which intranuclear amphophilic inclusion bodies (Cowdry type A) can be detected. In the pulmonary interstitium and peri-bronchial submucosa an heterophilic inflammatory infiltrate, often associated with lymphocyte and hyperemia and occasional hemorrhages is observed. In the liver there are multifocal areas of necrosis, often associated with included bodies from herpes similar to those observed in the lungs. In the spleen fibrinoid necrosis is present, associated with occasional viral inclusions at the endothelial level. From the same individual *Flavobacterium* spp. and *Chryseobacterium indologenes* were also isolated. [11]

Zoonotic potential: not reported.

Transmission: not known, but probably by direct contact. It is not known how long the virus can survive in water.

Distribution of the virus: at the moment the virus has only been identified in the United States.

Treatment: there is no treatment. The use of antibiotics is recommended to contain any secondary bacterial infections.

Differential diagnosis: lesions are similar to those induced by TeHV-3, however the difference between target species of tortoise (*Chrysemys picta*, the first and *Graptemys geographica*, the second) allows to distinguish the two viruses.

2.2 Emydid herpesvirus 2 (EmyHV-2)

Description/Overview: this virus has been identified exclusively in a molecular way, during a screening of a mixed bog turtle and spotted turtle population in the United States for conservational purposes. [20]

Signaling: the virus was identified in a small number of bog turtles (*Glyptemys muhlenbergii*) and in a single spotted turtle (*Clemmys guttata*).

Risk factors: are not known.

Molecular diagnostics: the virus can be identified through PCR.

Pathology: not known.

Zoonotic potential: not reported.

Transmission: not known but probably by direct contact. It is not known how long the virus can survive in water.

Distribution of the virus: at the moment the virus has only been identified in the United States.

Treatment: not known.

Differential diagnosis: not knowing the clinical profile of this pathology, it is not possible to establish a differential diagnosis.

3 *Glyptemys herpesvirus*es (GlyHV):

Description/Overview: this virus was identified exclusively in a molecular way, during the same screening in which *Emydid herpesvirus* 2 was identified. Two genotypes of this virus have been identified [20]:

1) GlyHV-1: exclusively in bog turtles (*Glyptemys muhlenbergii*)

2) GlyHV-2: exclusively in wood turtles (*Glyptemys insculpta*).

Signaling: the virus was identified in a small number of bog turtles (*Glyptemys muhlenbergii*) and wood turtles (*Glyptemys insculpta*).

Risk factors: not known.

Molecular diagnostics: the virus can be identified through PCR.

Pathology: not known.

Zoonotic potential: not reported.

Transmission: not known but probably by direct contact. It is not known how long the virus can survive in water.

Distribution of the virus: at the moment the virus has only been identified in the United States.

Treatment: not known.

Differential diagnosis: the clinical profile is unknown.

4 *Terrapene herpesvirus*es (TerrHV):
Description/Overview: This virus was identified for the first time in 2011 in eastern box turtles (*Terrapene carolina carolina*) held in captivity at two zoos in the south of the United States. In one of the two zoos no clinical signs were detected, while in the other one, about 46% of the individuals presented lethargy, stomatitis, cloacitis, conjunctivitis and blepharedema. In one of the zoos, the one characterized by the presence of gross lesions (see: "Pathology"), subjects were demonstrated to also be infected by ranavirus. [21, 22]

Signaling: the virus was identified in eastern box turtles (*Terrapene Carolina Carolina*).

Risk factors: not known.

Molecular diagnostics: the virus can be identified through PCR. [23]

Pathology: very variable. Generally, the presence of fibrinonecrotizing stomatitis and cloacitis, associated and conjunctivitis and blepharedema is detected. Unfortunately, the macroscopic finds are all compatible with a ranavirus infection. Histologically, the most common lesions are: presence pseudomembranes at the level of lungs and gums, esophagitis, gastritis, colitis, fibrinoid necrosis of the splenic vases, hepatitis with multi-focal areas of necrosis, interstitial nephritis and edema with pulmonary hyperemia. No herpesvirus associated intranuclear amphophilic inclusion bodies were identified in any of the organs or subjects examined. As for the gross lesions, the presence of stomatitis, esophagitis, hepatitis and splenitis has also been attributed to the presence of ranavirus in a population, it is therefore not clear exactly whether the lesions were induced by this virus. [21]

Zoonotic potential: not reported.

Transmission: not known but probably by direct contact. It is not known how long the virus can survive in water.

Distribution of the virus: at the moment the virus has only been identified in the United States.

Treatment: not known.

Differential diagnosis: not knowing the clinical profile of this pathology, it is not possible to establish a differential diagnosis. This disease must be included as differential for the ranavirus infection.

5 Testudinid herpesvirus (TeHV):

All herpesviruses recently isolated or detected in tortoises (*Testudinidae*) were grouped under the *Testudinid herpesvirus* (TeHVs) family. The first virus to be genetically characterized as part of this group was identified in an outbreak of mixed group of animals with russian turtles (*Agrionemys* [*Testudo*] *horsfieldii*), pancake turtles (*Malacochersus tornieri*) and greek turtles (*Testudo graeca*); this strain of herpesvirus is named TeHV-1 (*Testudinid herpesvirus* 1). Subsequently, in an outbreak in California, a herpesvirus was isolated with genetic characteristics different from TeHV-1; this virus is referred to as TeHV-2 (*Testudinid herpesvirus* 2). [24-26] Another strain was isolated from an individual belonging to a group of 16 *T. horsfeldii*, this had a lesion profile similar to those of TeHV1 and TeHV2, but some genetic differences were detected; this strain is called TeHV-3 (*Testudinid herpesvirus* 3). [27] In 2010 a

new genotype from Africa was then identified, called TeHV-4 (*Testudinid herpesvirus* 4). [28] It should be noted that some authors [28] relate to this virus with the acronym THV (THV-1, THV-2, THV-3, THV-4), or tortoise herpesvirus. The genotypes (serotypes viral) currently identified in as TeHVs are [28, 29]:

5.1 Testudinid herpesvirus 1 (TeHV-1)

Description/Overview: one of two genotypes that are assumed to originate in the Eurasian area. This genotype has a high incidence in *Agrionemys* [*Testudo*] *horsfieldii*, but has also been observed in other tortoise species. It is generally considered less virulent than genotype 3 (the second Eurasian genotype), but this conclusion is based only on anecdotic data. Because of the non-evident clinical signs, the *Agrionemys* [*Testudo*] *horsfieldii* is considered as vectors for this virus.

Signaling: the virus was identified in the *Agrionemys* [*Testudo*] *horsfieldii*, *Malacochersus tornieri*, *Testudo graeca* and *Testudo hermanni*. [29]

Risk factors: the presence of numerous turtles in confined spaces and non-compliance with the rules of quarantine.

Molecular diagnostics: The virus can be identified through PCR. [30]

Pathology: macroscopically, the virus causes the onset of necrotizing lesions of the oral cavity, pharynx and tongue, with the possible presence of diphthero-necrotic plaques. Less commonly, splenomegaly with ecchymosis and presence of pseudo membranes at the gastric level are also reported. Histologically, necrosis and fibrin at the level of the oral mucosa associated with the presence of heterophilic inflammation are commonly detected. Necrotic lesions may extend aborally to involve the cranial portion of the trachea. Less frequently necrotic lesions may also affect the liver, spleen, adrenals, kidneys, esophagus, duodenum, jejunum, colon and pancreas. Epithelial cells of the affected organs' intranuclear inclusion bodies can occasionally be found. [31]

Zoonotic potential: not reported.

Transmission: transmission can occur either through direct contact or via the environment.

Distribution of the virus: Europe.

Treatment: the use of antibiotics is recommended to prevent the onset of secondary bacterial infections.

Differential diagnosis: the main differential diagnosis is TeHV-3.

5.2 Testudinid herpesvirus 2 (TeHV-2)

Description/Overview: This genotype was detected only in *Gopherus agassizii* (desert tortoise) in the USA. [25] The lesions associated with this genotype are similar to those observed in the turtles infected with genotype 1 (TeHV-1) or 3 (TeHV-3).

Signaling: the virus was identified in desert tortoises (*Gopherus agassizii*).

Risk factors: the presence of numerous turtles in confined spaces and non-compliance with the rules of quarantine.

Molecular diagnostics: The virus can be identified through PCR and ELISA. [26] It is necessary to specify that the ELISA test used was developed for the identification of TeHV-3 and cross reacted with TeHV-2. [26]

Pathology: macroscopically the infection is characterized by the appearance of necrotizing lesions of the oral cavity, pharynx and tongue, with the possible presence of diphthero-necrotic plaques. Histologically, necrosis and fibrin at the level of the oral mucosa associated with the presence of heterophilic inflammation are observed in most of the cases. Less frequently, necrotic lesions may also involve other organs. The presence of intranuclear amphophilic inclusion bodies compatible with herpesvirus in epithelial cells is variable. [25]

Zoonotic potential: not reported.

Transmission: transmission can occur either through direct contact or via the environment.

Distribution of viruses: North America.

Treatment: the use of antibiotics is recommended to prevent the onset of secondary bacterial infections.

Differential diagnosis: the main differential diagnosis is with an infection by TeHV-3.

5.3 Testudinid herpesvirus 3 (TeHV-3)

Description/Overview: although considered the second Eurasian strain, TeHV-3 is the virus observed with greater frequency all over the world, and it is considered the most virulent of the four known genotypes up to now. The disease develops in the upper airways and in the cranial portion of the digestive tract. Clinically the infection is associated with the presence of nasal (from which it takes its origin, the classic English name "running nose") and oral discharge, which however cannot be considered specific findings. [29] The oral lesions are characterized by the formation of diphthero-necrotic plaques at the level of the tongue, which may extend to involve the esophagus and trachea. The severity of the injury is related to the infective dose of the virus. [29] *Testudo graeca* is, anecdotally, considered as vectors of TeHV-3.

Signaling: The virus was identified in *Testudo hermanni*, *Testudo graeca*, *Agryonemis* [*Testudo*] *horsfieldii*, *Testudo marginata* and *Testudo kleimnanni*. [4, 26]

Risk factors: the presence of numerous turtles in confined spaces and non-compliance with quarantine rules.

Molecular diagnostics: the virus can be identified through PCR and ELISA. [29]

Pathology: infected animals are largely characterized by the onset of necrotizing lesions of the oral cavity, pharynx and tongue, which are associated with the presence of diphero-necrotic plaques. Plaques may extend to involve the first tract of the digestive system. In *Testudo graeca* necrotic plaques may extend to involve the tracheal bifurcation. If the infective dose of virus is low, the subject may survive the viraemia; this is associated with the vanishing of the oral plaques within 24 days with consequent, complete, recovery of the mucosa. Hepatomegaly and edema of the head are other signs reported in the literature. [29] Histologically, necrosis and fibrin at the level of the cranial portions of digestive and respiratory tracts are commonly described. The presence of amphophilic intranuclear inclusion bodies compatible with herpesvirus in epithelial cells is variable, and depends on the timing of the lesions. Usually,

inclusion persists for three weeks after the onset of viremia. [29] Secondary bacterial or viral infections are not uncommon.

Zoonotic potential: not reported.

Transmission: transmission can occur either through direct contact or via the environment.

Distribution of the virus: worldwide.

Treatment: the use of antibiotics is recommended to prevent the onset of secondary bacterial infections. Aciclovir has proved effective in the containment of the clinical signs.

Differential diagnosis: the main differential diagnosis includes TeHV-1, Iridovirus (Ranavirus), picornaviruses (virus "X") and Mycoplasma spp. TeHV-2 must be considered as differential if the animals are imported from the US.

5.*4 Testudinid herpesvirus 4* (TeHV-4)

Description/Overview: the virus was reported for the first time in two wild caught angulate tortoises (*Chersina angulata*) from South Africa that were exported to the USA. Both animals were asymptomatic and the virus was identified during the import controls. [28] More recently (2016), TeHV-4 has been reported in a leopard turtle (*Stygmochelys pardalis*) in a private collection in Germany, which exhibited respiratory distress. [28, 32] However, it is to be pointed out that the subject was co-infected with *Mycoplasma* spp. and that the clinical signs reported may be attributable to the bacterial infection.

Signaling: the virus was identified in the angulate tortoise (*Chersina anulata*) and in a leopard tortoise (*Stygmochelys pardalis*).

Risk factors: the presence of numerous turtles in confined spaces and non-compliance with quarantine rules.

Molecular diagnostics: the virus can be identified through PCR. [28, 32]
Pathology: not reported.

Zoonotic potential: not reported.

Transmission: can occur either through direct contact or via the environment.

Distribution of the virus: the virus was only identified in turtles from Africa, but is probably distributed world-wide.

Treatment: the use of antibiotics is recommended to prevent the onset of secondary bacterial infections.

Differential diagnosis: without a true lesion profile, only the differential diagnosis remains *Mycoplasma* spp.

Discussion:

Thus, the amount of information is progressively increasing, most of the biology and host-pathogen interaction of most of chelonian's herpesvirus is still unclear or underinvestigated. The understanding of *Chelonivirus* genomics and host-pathogen interaction will lead to develop new therapeutic approaches allowing veterinarian to treat these lethal diseases and promote the conservation of turtles and tortoises worldwide.

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Chapter 5: Detection of TeHVs in Italy.

Introduction:

The presence of tortois herpesvirus in Italy has only been marginally investigated. In 2003 Origgi et al. [1], detected the presence of an outbreak of tortoise herpesvirus in Italy, more recently (2017), Marschang et al. [2], found no herpesvirus positive samples from 11 tortoise sera submitted from Italy to a German laboratory.

Although little work has been done to detect the presence of tortoise herpesvirus in Italy, signs correlating with this infection are constantly reported by Italian veterinarians.

To verify the presence of the virus in Italy, we decided to use three different approaches:

1) We started monitoring the tortoise population of a WWF oasis located in Northern Italy, which is responsible for collecting wildlife and abandoned exotic animals and pets from the north-west of the country.

   The oasis manager stated that since 2010, the *Testudo* spp. population housed in the WWF wildlife rescue center of Vanzago has started exhibiting various clinical signs of severe lethargy, nasal discharge, conjunctivitis, and diphtheronecrotic glossitis. During that period 50 tortoises died, with reported mortality peaks during March and October. In Spring 2014, the Vanzago center's population consisted of 9 *T. marginata*, 7 *T. hermanni* and 2 *T. graeca*, still variably exhibiting the same, abovementioned clinical signs, which were compatible with a herpesvirus infection in these species.

   To verify our hypothesis, in late spring 2014 we collected serological samples from the affected animals, which we used to perform ELISA tests. All spontaneously dead tortoises were submitted to the Department of Veterinary Medicine of the University of Milan for routine necropsy. By the end of 2015 all animals had died.

2) During our routine necropsy, both on Vanzago's and external cases, macroscopic and microscopic lesion profiles compatible with TeHVs infection were noted on a specific data sheet, and for each case tissue samples for molecular biology and histopathology were collected.

3) To increase our sample size and understand the history of TeHVs in Italy, we also decide to include retrospective samples from tortoises examined from 1999 to 2013.

Material and methods:

Serological screening:

The tortoises' blood was collected by jugular venipuncture with a sterile, 29G, and 12.7 mm long insulin needle. Before and after venipuncture the skin area was disinfected with a 10% Povidone-iodine solution.

One hundred-fifteen microliters of blood were collected from each animal but one, which was too small (from a total of 17 animals).
One milliliter lithium-heparin test tubes were used to collect and store the blood at +4°C. Later on, blood was centrifuged at 350g for 5 minutes at room temperature and frozen at -80°C until analyses were performed.

ELISA test:

Viral antigen was obtained by infecting tortoise tissue cultures, namely *Terrapene* Heart 1 (TH-1). To obtain the viral antigens the protocol described by Origgi et al. (2003) [1] was used. Briefly, once the onset of cytopathic effects was detected, cell cultures were consecutively frozen and thawed three times. The obtained solution was centrifuged at 4,500g for half an hour for three consecutive times to precipitate cellular debris.
The purified supernatant was centrifuged one more time at 53,644g for 3.5 hours at 4°C to allow viral particles to pellet. The viral pellets were collected, and resuspended in test tubes containing a continuous sucrose gradient ranging from 20% to 60% in TNE (100 mM Tris, 2 M NaCl, 10 mM EDTA, pH 7.4). Viral particles were centrifuged at 156,194g for 2 hours at 4°C, obtaining nine viral fractions. Viral concentration was estimated for each fraction using three different techniques:

- Protein assay
- Speed of onset and severity of cytopathic effects in TH-1 tissue cultures
- Negative-straining electron microscopy (NEM)

Among the nine fractions, only two were considered pure and concentrated enough to be used for antigen preparation. The two selected were than centrifuged at 53,644g for 3.5 hours at 4°C. The obtained viral pellets were than suspended in 7.2 pH PBS solution and stored at -80°C.
ELISA tests were performed using 96 well (Sigma-Aldrich, Milan, Italy) coated with viral antigen. Each well was prepared using 50µl of 0,001 M PBS-A (0.15 M NaCl plus 0.02% of N_3Na, with 7.2 pH) solution containing 5µg/ml of viral antigen, and incubated overnight at 4°C.
The morning after, wells were washed four times each with PBS-T (PBS-A plus 0.05% Tweed 20), following that antigen blocking was performed, using 5% skimmed milk PBS-A solution incubated at room temperature for 60 minutes. Protein biotinylation was performed using PBS-A solution containing 1µg/ml biotin (Mab HL1546) incubated for 60 minutes at room temperature, wells were than washed four times with PBS-A.

Each well was filled with 50µl Streptavidin conjugated alkaline phosphatase for 90 minutes, and then washed four times with PBS-T. One hundred microliters of p-nitrophenyl disodium phosphate solution (1mg/ml of p-nitrophenyl diluted in a 0.01M bicarbonate solution [pH 9.6] with 2 mM $MgCl_2$) were added to each well and incubated in the dark for 90 minutes, to allow the colorimetric reaction.
Colorimetric reaction intensity was evaluated using an ELISA plate reader (MTX Lab system, Spectramax 190) at 405 nm of absorbance.

Necropsy protocol:

Necropsy was performed on 28 tortoises. Eighteen were from Vanzago, subdivided into 7 *Testudo hermanni*, 9 *Testudo marginata*, and 2 *Testudo graeca*. Among the remaining ten routine cases, only those coming from the same geographical area of Vanzago (North-West Italy) were included in the study, the reason for this choice was to have a more homogenous distribution of the sample. Ten external cases fitted the requirements, and were subdivided into 1 *Testudo graeca*, 5 *Testudo hermanni hermanni*, 3 *Testudo hermanni botgeri* and 1 *Testudo horsfieldii*.
To standardize the necropsy data collection, a printable form that could be filled online was created in Google Drive (Google inc. ©) (See Annex I).
All examined animals had died spontaneously, and owner authorization was requested before necropsy. Unfortunately, most of the cases were frozen before submission to the Veterinary anatomic pathology unit of the Department of Veterinary Medicine of the University of Milan.
All the submitted animals had a history of lethargy with abnormal hibernation behavior (late onset, multiple wake-ups during the hibernation season, and unusual burrowing), and some of them were reported to have ocular and nasal discharge.
For each of the examined cases the following parameters were recorded:

1. Morphological parameters (species, gender, weight, width, length). To assess the right species the CITES guideline for tortoise identification was used [3].
2. Necropsy findings
3. Histological findings

Before necropsy, each animal underwent an oral swab that was placed in a 2.0 ml sterile cryovial tube containing DMEM. Due to the small size of the animals and the resistance opposed by the mandibular musculature, surgical forceps immersed for 30 minutes in a 6 volumes sodium hypochlorite solution (NaOCl) were used to keep the mouth open.

The swab was performed avoiding contact with oral cavity surfaces except for the tongue, and then stored in a 2ml Eppendorf tube containing Dulbecco's Modified Eagle Medium (DMEM – ThermoFisher, Milan, Italy) and cell culture antibiotic solution at 4°C for less than one month. During necropsy, organs were collected for both histopathology and molecular biology. Because of the poor conservation status, three tortoises (*Testudo hermanni hermanni*) from Vanzago's oasis (PC 184/14, PC 197/15, PC 198/15) were excluded from the histopathology study, but samples were included anyway in the molecular biology evaluation.
All tortoises were opened and examined for the presence of gross lesions, which were noted on the form (Annex I). Samples for histopathology were collected in formalin proof buckets and stored one week before processing for histopathology.
Two 5x5 mm fresh tissue sections were collected during necropsy for molecular biology. Samples were stored in sterile 2.0 ml cryovials (Nalgene, New Jersey, USA), one with and one without DMEM, and frozen at -20°C until the diagnostic procedures were carried out.
The samples collected for both histopathology and molecular biology were brain, tongue and pharynx, trachea, esophagus, liver, lungs, stomach and intestine, genital system, kidneys, and heart. The carapace was collected for histology only.
After one-week fixation in 10% buffered formalin (Sigma-Aldrich, Milan, Italy) tissue samples were processed for histopathology, except for the carapace that was demineralized for one week in a 10% formic acid solution; the acidity was then quenched by one hour of immersion in tap water. Due to the heavy keratinization of tortoise shells, demineralized samples were also immersed in a 50% solution of Marseille soap for three days before paraffin inclusion.

Prospective collection protocol:

The formatted fixed samples were routinely processed, subsequently paraffin was included. From each sample, a 5m section was placed on a standard histology slide. Thicker sections (6-7 μm) were sliced for harder to cut tissues (such as bone or intestine) and placed on histology glue auditioned slides (poly-lysine coated slides).

Sections were standardly stained with Haematoxylin-Eosin; additional histochemical stains, such as Gram, PAS, Ziehl-Neelsen, and Fite-Faraco were performed when needed.

Retrospective sample collection protocol:

To increase the sensibility and significance level of the study about the prevalence of TeHVs (*Testudinid herpesvirus*) in Italy, retrospective samples of tortoises were selected from the Veterinary anatomical pathology archive of the Department of Veterinary Medicine of the University of Milan.

Samples were retrieved from the electronic archive using the following keyword combinations: “tortoises”, “herpes”, “diphthero-necrotic plaques”, “stomatitis”, “fat-bodies atrophy”, “mucopurulent discharge”, and ulcerative glossitis”.

The case selection was further refined using the following criteria:

1. Animals that according to the necropsy or histopathology report were strongly suspected of a herpesvirus infection, either because of classic intranuclear inclusion bodies or diphthero-necrotic lesions of the oral cavity.
2. Animals that had gross or histological lesions that were compatible with, but not classically associated with a herpesvirus infection.

To avoid possible contamination between routinely processed histology cassettes and the selected retrospective samples, one of the old microtomes of the Department of Veterinary Medicine of the University of Milan was designated for PCR sample preparation and placed in a different room.

Microtome cleaning procedures consisted of rubbing all the visible surfaces with acetone and hydrogen peroxide before starting the procedure, and after processing each sample. The combination of the reagents guaranteed the inhibition of the viral particles and made it easier to remove paraffin residue before processing a new histology block. To avoid contamination as far as possible, a single microtome blade was used for each histology block.

From each retrospective case, a 30 μm slice was obtained and stored in a sterile, 2 ml, RNase-free cryovial (Nalgene, New Jersey, USA). Cryovials containing retrospective samples were closed and stored at room temperature until processed.

Most of the retrospective cases were surgical pathology samples or necropsy in a bottle comprising only a few organs, with no macroscopic descriptions.

Samples included in the study are reported in the table below (Table 1):

PROTOCOL NUMBER	HISTOLOGICAL DIAGNOSIS
EX 6/12	SUSPECT HERPESVIRUS INFECTION
EX 33/12	SUSPECT HERPESVIRUS INFECTION
EX 34/12	SUSPECT HERPESVIRUS INFECTION
EX 23/12	CATARRHAL STOMATITIS
EX 179/10	SEPTICEMIA WITH FUNGAL HYPHAE
EX 169/10	SEPTICEMIA WITH INTRAVASCULAR BACTERIA
EX 66/10	*HERPESVIRUS INTRANUCELAR INCLUSIONS*
EX 137/08	SUSPECT HERPESVIRUS INFECTION
EX 122/08	SUSPECT HERPESVIRUS INFECTION
EX 96/08	SUSPECT HERPESVIRUS INFECTION

Table 1: Retrospective samples selected from the archive to be PCR tested for TeHVs because of the lesion profile. Cells with a light-yellow background are samples with originally suspected herpesvirus infection.

PROTOCOL NUMBER	HISTOLOGICAL DIAGNOSIS
EX 67/05	NECROTIZING HEPATITIS
EX 221/04	HETEROPHILIC TRACHEITIS AND PNEUMONIA
EX 17/04	GRANULOMATOUS HEPATITIS OF UNKNOWN ORIGIN
EX 184/02	GRANULOMATOUS HEPATITIS OF UNKNOWN ORIGIN
EX 27/02	LIVER LIPIDOSIS WITH INCREASED NUMBER OF MELANO-MACRO-PHAGE CENTERS
EX 12/00	LEUKEMYA
EX 32/00	HETEROPHILIC RHINITIS
EX 43/00	UKCREATIVE DERMATITIS OF UN-KONWN ORIGIN
EX 45/00	HETEROPHILIC HEPATITIS

Table 1 (continued): Retrospective samples selected from the archive to be PCR tested for TeHVs because of the lesion profile. Cells with a light-yellow background are samples with originally suspected herpesvirus infection.

PCR protocol:

Two different PCR protocols were used to detect herpesvirus DNA in both prospective and retrospective samples.

- Pan-herpes protocol:

  The Pan-herpes PCR protocol was first developed by VanDervanter et al. (1996), and can detect a very conserved region of the herpesvirus DNA-polymerase, allowing to detect the presence of a wide range of herpesviruses. Briefly, this a nested PCR using two sets of primers containing degenerate nucleotides. Degenerate nucleotides are synthetic nucleotides that do not respect the classic base pairing (C-G, A-T), thus allowing the PCR probe to bind to template DNA sequences that present variations in the nucleotide sequence.

  Standard and degenerate nucleotides are reported in Table 2.

Une et al. (1999), Origgi et al. (2004), and Kolesing et al. (2016) [2-4] demonstrated that this PCR protocol is able to amplify the DNA polymerase sequence of all known TeHVs genotypes.

Primers used for the first amplification step:

1) DFA – 5' - GAYTTYGCNAGYYTNTAYCC-3';
2) ILK – 5' - TCCTGGACAAGCAGCARNYSGCNMTNAA-3';
3) KG1 – 5' -GTCTTGCTCACCAGNTCNACNCCYTT-3';

Primers used for the second amplification step:

1) TGV – 5' -TGTAACTCGGTGTAYGGNTTYACNGGNGT-3';
2) IYG – 5' -CACAGAGTCCGTRTCNCCRTADAT-3';

Amplification steps 1 and 2 used to detect herpesvirus in our samples are reported in Table 3 and Table 4 respectively.

UPAC nucleotide code	Base	Notes
A	Adenine	
B	C or G or T (U)	This degenerate nucleotide does not bind A
C	Cytosine	
D	A or G or T (U)	This degenerate nucleotide does not bind c
G	Guanine	
H	A or C or T (U)	This degenerate nucleotide does not bind G
K	G or T (U)	Ketoses
M	A or C	Amine
N	All the possible bases	The letter N comes from any
R	A or G	Purines

Table 2: Synthetic and natural nucleotide list, in alphabetical order.

Step 1:

	µl		
2x Qiagen Hot Star MM	12.50		
DFA	0.25	1.0 µM	
ILK	0.25	1.0 µM	
KG1	0.25	1.0 µM	
ng	*		
H_2O	*		
	25.00		
Cycling	15 min.	95ºC	Hotstar Activation
	30 sec.	95ºC	45x
	1 min.	46ºC	
	1 min.	72ºC	
	10 min.	72°C	Extension
	4ºC ∞		

Table 3: TeHV3 step 1 amplification protocol.

Step 2:

	µl		
2x Qiagen Hot Star MM	12.50		
TGV	0.25	1.0 µM	
IYG	0.25	1.0 µM	
DNA of 1° PCR	2.50	1.0 µM	
H2O	9.50		
	25.00		
Cycling	15 min.		
		95ºC	Hotstar Activation
	30 sec.		
	1 min.	95ºC	
	1 min.	46ºC	45x
		72ºC	
	10 min.		
		72°C	Extension
	4ºC ∞		

Table 4: TeHV3 step 2 amplification protocol.

- Semi-quantitative PCR for TeHV3 protocol:
  TeHV3 PCR was carried out according to the protocol established by Origgi et al. (2003). [1] This PCR amplified the partial sequence of the gene homologous to the ribonucleotide reductase (UL39 homologous gene).
  Two primers were used to amplify the sequence:
  1) THV1 –5′-TGCACTTTGATGCGTGGGAT-3′.
  2) THV2 –5′-TTGATCGTATTCGAATGCCG-3′
  The amplification protocol is reported in Table 5.

	µl		
2x Qiagen Hot Star MM	12.50		
THV1F	0.50	1.0 µM	
THV2R	0.50	1.0 µM	
DNA	2.50		
H_2O	9.00		
	25.00		
Cycling	15 min.	95ºC	Hotstar activation
	1 min.	95ºC	35x
	30 sec.	54ºC	
	30 sec.	72ºC	
	10 min.	72ºC	Extension
	4°C∞		

Table 5: PCR amplification protocol for TeHV3.

Both PCR protocols were carried out using a standard PCR thermocycler (DNAengine, MJ Research, Waltham, MA, USA). Each PCR was run with both negative and positive control to verify the correct PCR implementation and the possible presence of contamination.
Samples that tested negative for TeHV3 but positive for the PanHerpes PCR were also tested for the presence of TeHV1, TeHV2, and TeHV3 according to the protocol suggested in literature [4-6].

Nucleotide titering:

The total concentration of DNA extracted from both fresh and paraffin embedded samples was titered using absorbance and fluorescence (Nanodrop, ThermoFisher, Ecumblens, Switzerland) simultaneously.

Electrophoresis:

The presence of amplification bands was verified by mixing 2µl of each PCR product with 0.5 µl of loading dye (Thermo Scientific, Switzerland) and 0.5 µl of Red-Safe (Applied Biological Materials, Vancouver, Canada) and then loaded on a 2% Agarose gel. Gels were loaded in an electrophoresis machine (Bio Rad, Hercules, CA, USA) and run for 90 minutes at 85V. Both negative and positive controls were always loaded on each gel.

Tissue cultures:

TeHV virions were grown on *Terrapene* Heart 1 (TH-1, American Type Culture Collection [ATCC], Rockville, MD, USA) cell line. TH-1 is a cardiomyocytes cell line obtained from the heart of box turtles (*Terrapene carolina carolina*). [7]

TH-1cell line requires a temperature of 28°C with 5% CO2 to adequately replicate and grow. TH-1 cell stock stored at -80C was defrosted, partitioned and placed in small flasks (Green house, Baton Rouge, LA, USA) of 9cm x 5cm x 2,5cm (25cm³) to increase cells replication speed. To each vial, a total volume of about 250x103 cells with 5ml of DMEM (Life technologies, Zug, CH) was added.

Once tissue culture monolayer was obtained, cells were split and transferred into 75cm³ flasks (Green house, Baton Rouge, LA, USA).

The following procedure was used to split the cells:

1) The DMED was removed from the flask using a sterile pipet tip
2) Three washes with sterile PBS were made
3) Zero point five milliliters of sterile and cold trypsin were poured into the flask
4) The flask with trypsin was incubated at room temperature for 3-5 minutes, to allow cells to detach from the flask floor
5) Five milliliters of fresh DMEM with Bovine Sero-Albumin was added to the flask to stop trypsin activity and resuspend cells.
6) The DMEM with cells in suspension was transferred to a new flask
7) Another 5ml of fresh DMEM was added to the flask
8) Flasks were then incubated as previously described

Viral isolation:

Although todays' molecular techniques allow to rapidly identify a virus with a very high sensitivity and specificity, these techniques do not work on viral particles. The main advantages of using viral isolation are:

- Verify the presence and the virulence of a virus
- Have a large number of virions to perform other tests like ELISA or DNA sequencing

Previously collected tissue samples and oral swabs added with DMEM were used to perform viral isolation.

Cryovial content was extracted in sterile mortar and reduced to paste with the use of a sterile pestle and quartz sand. Once tissue destruction was completed, fresh DMEM was added to the mortar until a viscous solution was obtained. The obtained solution was transferred to a 15 ml Falcon tube and centrifuged for 10 minutes at 1500g and 4°C (Thermo/Heraeus Multifuge 3SR+, Osterode, D). Using a 50ml sterile syringe the supernatant, without quartz sand, was transferred to a new sterile15ml Falcon tube.

According to standard procedures, half of the supernatant was filtrated using a 2cm in diameter, glass fiber syringe filter with 0.45μm wide pores (Sigma-Aldrich, Buchs, Switzerland), connected to a 5ml syringe. The other half was not filtrated.

Filtrated and non-filtrated supernatant were diluted 1:10 with fresh DMEM with no bovine Sero Albumin to which cell cultures in antibiotic solution were added (Ciprofloxacin, Sigma-Aldrich, Buchs, Switzerland).

Two milliliters of each supernatant dilution were added to separate cell culture flasks containing a monolayer of Terrapene Heart 1 (TH-1, American Type Culture Collection [ATCC], Rockville, MD, USA) close to confluence (80% of the flask).

After one hour of incubation at 25°C (room temperature), flasks were washed three times with sterile PBS, and 5ml of a solution containing sterile DMEM, Ciprofloxacin (Sigma-Aldrich, Buchs, Switzerland) and 10% Bovine Sero-Albumin was added. The flasks were then placed in a cell culture incubator at 28°C with 5% CO_2 and monitored daily.

Results:

Serological screening:

Seventeen of the 18 animals present in the Oasis were ELISA tested for the presence of TeHV3. One of the animals was excluded from the study because it was too small to collect enough serum to perform the ELISA test.

According to the information provided by Origgi et al. (2003) [1], the ELISA test positivity cut-off was set to an optical density of 0.48.

Sixteen of the 17 tested animals were seropositive for TeHV3, for a total TeHV3 seroprevalence in the Oasis of 94.73%. The only negative ELISA test was from a *T. hermanni* (case one, identification number 14347) with an optical density of 0.1.

Results of all the ELISA tests are reported in Table 6.

CASE #	IDENTIFICATION CHIP	TORTOISE SPECIES	OPTICAL DENSITY (O.D.)
1	14347	*Hermanni*	0.1
2	2p	*Hermanni*	2.4
3	17604	*Greca*	1.2
4	Not detected	*Marginata*	2.6
5	09259	*Marginata*	2.6
6	13928	*Marginata*	2.7
7	17461	*Marginata*	2.6
8	T1H-1p	*Hermanni*	0.51
9	11389	*Hermanni*	0.98
10	17593	*Hermanni*	0.54
11	15516	*Marginata*	2.8
12	18603	*Marginata*	2.8
13	17425	*Maginata*	3.0
14	16020	*Hermanni*	0.48
15	T1H-2p	*Hermanni*	0.8
16	10989	*Greca*	1.3
17	09474	*Marginata*	4.0

Table 6: ELISA test results for Vanzago's Oasis tortoises. Positivity cut-off was set at 0.48, based on the parameters reported by Origgi et al., 2004. The sample with a light-yellow background was the only negative, among the tested animals.

Mean optical density value obtained for each tortoise species recorded in the study is reported in Table 7.

SPECIES	MEAN O.D. VALUE	NUMBER OF EXAMINED TORTOISES
Testudo marginata	2,92	9
Testudo hermanni	0,83	6
Testudo graeca	1,25	2

Table 7: Mean optical density value (O.D.) obtained for each examined tortoise species.

Necropsy:

Necropsy were performed on a total of 28 tortoises, 18 from Vanzago's oasis and 10 animals submitted from the North-East of Italy for routine diagnostics. The examined population consisted of 37.04% females and 62.97% males. The mean age was extremely variable, from a few months up to ten years.

The species were distributes as follows: 12 *Testudo hermanni hermanni, 9 Testudo hermanni boettgeri, 4 Testudo marginata, and 3 Testudo graeca.* A summary of the main phenotypical characteristics of the examined tortoises is presented in Table 8.

CASE #	IDENTIFICATION PROTOCOL	SPECIES	GENDER	LENGTH (cm)	WIDTH (cm)	WEIGHT (g)
1	PC 184/14	*Testudo hermanni hermanni*	M	14	12	102
2	PC 185/14	*Testudo hermanni hermanni*	F	17	12	430.8
3	PC 186/14	*Testudo hermanni hermanni*	M	12	10	76.3
4	PC 191/14	*Testudo graeca*	F	23	15	800
5	PC 192/14	*Testudo hermanni hermanni*	M	14	10	228.74
6	PC 193/14	*Testudo hermanni hermanni*	M	20	13	500
7	PC 226/14	*Testudo hermanni boettgeri*	M	18	15	172.35
8	PC 227/14	*Testudo graeca*	F	14	12	120.4
9	PC 112/15	*Testudo hermanni hermanni*	M	12	8	98
10	PC 113/15	*Testudo hermanni boettgeri*	F	12	10	102
11	PC 114/15	*Testudo hermanni boettgeri*	M	17	12	450
12	PC 127/15	*Testudo marginata*	F	16	12,3	310
13	PC 134/15	*Testudo marginata*	M	25	17	819
14	PC 135/15	*Testudo marginata*	M	17	12	203
15	PC 136/15	*Testudo boettgeri*	M	15	12	205
16	PC 197/15	*Testudo hermanni boettgeri*	M	20	14	483

Table 8: Main phenotypical characteristics of tortoises that underwent necropsy during the study.

CASE #	IDENTIFI-CATION PROTOCOL	SPECIES	GEN-DER	LENGTH (cm)	WIDTH (cm)	WEIGHT (g)
17	PC 198/15	*Testudo hermanni boettgeri*	F	18	15	429
18	PC 199/15	*Testudo hermanni sp.*	M	21	16	495
19	EX 116/14	*Testudo graeca*	M	27	18	550
20	EX 90/15	*Testudo marginata*	F	16	12.5	500
21	EX 114/15	*Testudo hermanni hermanni*	M	15	10	300
22	EX 126/16 A	*Testudo hermanni hermanni*	F	15	9	300
23	EX 126/16 B	*Testudo hermanni hermanni*	M	10	7	230
24	EX 126/16 c	*Testudo hermanni boettgeri*	F	17	12	450
25	EX 149/16	*Testudo hermanni boettgeri*	M	20	17	600
26	EX 156/16	*Testudo hermanni boettgeri*	F	24	18	800
27	EX 191/16	*Testudo hermanni hermanni*	M	17	13	450
28	EX 24/17	*Testudo hermanni hermanni*	M	21	15	600

Table 8 (Continued): Main phenotypical characteristics of tortoises that underwent necropsy during the study.

The most common gross findings on severity and distribution are recorded in Table 9. Lesion severity and distribution recorded in Table 9 is expressed as a score ranging from 1 to 5, the score legend is reported below:

LESION SEVERITY:

1. Mild
2. Moderate
3. Moderate to Severe
4. Severe
5. Most severe

LESION DISTRIBUTION:

1. Focal
2. Multifocal (<5 lesions)
3. Multifocal (≥5 lesions)
4. Scattered
5. Diffuse

ORGAN SYSTEM	MEAN SEVERITY	MEAN DISTRIBUTION	MEAN PREVALENCE
GENITAL SYSTEM	3.3	2.5	37.5%
FAT-BODIES	3	3	13%
SKIN	1	1	13%
ESOPHAGUS	1	1	13%
LIVER	3	3	13%
TONGUE AND PHARINX	1.7	2.3	37.5%
EYES	1	1	13%
KIDNEYS	3	2	13%
STOMACH AND GUT	3.5	4	25%

Table 9: Mean lesion severity, distribution, and prevalence of the most common gross findings identified during necropsy.

Histopathology:

Prospective samples:

The presence of severe histological autolytic changes not detectable grossly did not allow to evaluate histopathological changes on individual organs or entire bodies.

Among the cases from Vanzago's Oasis, three bodies were considered severely autolytic at the necropsy (PC 184/14, PC 197/15, and PC 198/15) and other two cases were excluded during the histological evaluation because more than 70% of the organ systems routinely evaluated were histologically unreadable (PC 191/14, PC 193/14), for a total of five cases. Excluding the cases mentioned above, the total number of histological cases from Vanzago was 13. Autolytic changes in routine cases (EX116/14, 90/15, EX114/15, EX126/16 A/B/C, EX149/16, EX156/16, EX191/16, and EX24/17) were not so severe, and all the tortoises were included in the histological evaluation.

The overall histopathologically evaluated cases from both Vanzago and the necropsy service was 23 (28 selected cases, subtracting 3 autolytic bodies and 2 histologically unreadable cases).

The most commonly encountered histological change was the presence of bacteria in the trachea, lungs, esophagus, and coelomic cavity, frequently associated with an heterophilic and macrophagic inflammatory infiltrate.

Most commonly TeHVs associated injuries was heterophilic inflammation of tongue, gum, and trachea. Frequently tongue chorion inflammation was associated with mucosal ulceration, necrosis, and hemorrhages.

Heterophilic granulomas of the liver and kidneys were not an uncommon finding. All the granulomatous lesions were negative for the presence of fungi (PAS stain), mycobacteria (Ziehl-Neelsen), and atypical mycobacteria (Fite-Faraco).

Evaluation of histopathology as screening technique on prospective samples*:*

Histopathology is a routine diagnostic technique in veterinary pathology, whereas molecular and serological investigations are not as widespread as would be desired. To better complement the sensitivity and specificity of histopathology to detect TeHVs infections, two tables were prepared. Table 10 contains results of TeHV3 PCR subdivided by case and organ. Table 11 contains detected histological lesion compatible with TeHVs infection. Both tables' results are expressed with a score ranging from 0 to 2.

PCR results listed in Table 11 are expressed with the following grading system:

0) Negative
1) Mild positivity
2) Strong positivity

TeHV3 related or possibly related histological lesions are expressed as follows:

0) No TeHV3 indicative lesion
1) TeHV3 suggestive lesions
2) Presence of herpesvirus associated intranuclear amphophilic inclusion bodies
-) Not evaluable because of the severe autolytic changes

	PC 184/14	*PC 185/14*	*PC 186/14*	*PC 191/14*	*PC 192/14*	*PC 193/14*	*PC 226/14*	*PC 227/14*	*PC 112/15*	*PC 113/15*	*PC 114/15*	*PC 127/15*	*PC 134/15*	*PC 135/15*	*PC 136/15*	*PC 197/15*	*PC 198/15*	*PC 199/15*	*EX 116/14*	*EX 90/15*	*EX 114/15*	*EX 126/16 A*	*EX 126/16 B*	*EX 126/16 C*	*EX 149/16*	*EX 156/16*	*EX 191/16*	*EX 24/17*
BRAIN	*0*	*2*	*2*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
TONGUE AND PHARINX	*0*	*2*	*0*	*0*	*2*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*
TRACHEA	*2*	*2*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
ESOPHAGUS	*0*	*2*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LI-VER	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LUNGS	*0*	*2*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
STOM-ACH AND IN-TE-STI NE	*0*	*2*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
GE-NI-TAL AP-PA-RA-TUS	*1*	*1*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KID-NEYS	*0*	*1*	*2*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
HEART	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

Table 10: Results of TeHV3 PCR screening on examined tortoises. PC = samples from Vanzago, EX = routine necropsy samples.

	PC 184/ 14	*PC 185/ 14*	*PC 186/ 14*	*PC 191/ 14*	*PC 192/ 14*	*PC 193/ 14*	*PC 226/ 14*	*PC 227/ 14*	*PC 112/ 15*	*PC 113/ 15*	*PC 114/ 15*	*PC 127/ 15*	*PC 134/ 15*	*PC 135/ 15*	*PC 136/ 15*	*PC 197/ 15*	*PC 198/ 15*	*PC 199/ 15*	*EX 116/ 14*	*EX 90/ 15*	*EX 114/ 15*	*EX 126/ 16 A*	*EX 126/ 16 B*	*EX 126/ 16 C*	*EX 149/ 16*	*EX 156/ 16*	*EX 191/ 16*	*EX 24/ 17*
BRAIN	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
TONGUE AND PHARINX	-	*0*	*0*	-	*2*	-	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*0*	-	-	*0*	*2*	*0*	*1*	*0*	*0*	*2*	*0*	*0*	*1*	*1*
TRACHEA	-	*0*	*2*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*1*	*0*	*1*	*0*	*1*	*0*	*0*	*2*	*0*
ESOPHAGUS	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LI-VER	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LUNGS	-	*0*	*1*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
STOMACH AND IN-TE-STINE	-	*0*	*0*	-	*0*	-	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*1*	*0*	*0*	*1*	*1*	*1*	*1*	*0*	*0*	*0*
GE-NI-TAL AP-PA-RA-TUS	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KID-NEYS	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
HEART	-	*0*	*0*	-	*0*	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	-	-	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

Table 11: Results of TeHV3 histology screening on examined tortoises. PC = samples from Vanzago, EX = routine necropsy samples., - = samples excluded from the histological evaluation because of the poor conservation status.

Summarizing, there were eight PCR positive animals from Vanzago (PC 184/14, PC 185/14, PC 186/14, PC 191/14, PC 192/14, PC 193/14, PC 134/15, and PC 135/15) and four positive routine PCR samples (EX 116/14, EX 90/15, EX 114/15, EX 24/17), for a total of 12 positive animals from the 28 examined.

Retrospective samples:

To increase the sample size, retrospective cases ranging from 1999 to 2012 were selected from the veterinary anatomical pathology section digital archive. Digital archive screening using the keywords reported in the material and methods section returned 60 tortoise samples, subdivided into 23 necropsies and 37 surgical specimens.

Selection criteria were then refined considering the presence of:

- Lesions supportive for immunosuppression (e.g. bacterial overgrowth, spleen white pulp involution)
- Lesions supportive of a TeHV3 infection
- Intranuclear inclusion bodies in key organs like tongue or the respiratory tract epithelium.

This further selection reduced the case load to 27 samples, classified as follows:

- Two animals with intranuclear inclusion bodies (EX 6/12 and EX 66/10)
- Ten animals with lesions classically associated with TeHVs infections
- Fourteen animals with lesions occasionally associated with TeHVs infections
- Two animals with neoplastic lesions.

No literature is available about the possible role of TeHVs in inducing neoplastic disease in tortoises, contrary to other herpesviruses in reptiles (e.g. *Chelonian herpesvirus* 5), birds (Marek's disease, *Gallid herpesvirus* 2) and mammals (*Samiri herpesvirus* 1). These two samples were included to check if the animals were also TeHVs positive.

The gross inclusion criteria included: diphtheronecrotic glossitis and gingivitis, nasal discharge, and liver granulomas.

The histological inclusion criteria included: liver and kidney granulomatous lesion, heterophilic inflammation of the tongue, trachea, lungs, and liver, septicemic diseases, severe intestinal oxyurid proliferation, ulcerative skin diseases, abscesses, and degenerative liver diseases.

The necropsies of four surgical specimens fitted the macroscopic and microscopic inclusion criteria, namely EX 34/12, EX 33/12, EX 6/12 and EX 66/10.

Based on the selection criteria, twenty-three surgical cases were included in the study.

Most common organs submitted as surgical samples were (in descending order): digestive tract (63%), lungs (60%), liver (57%), trachea and larynx (53%), genital tract (10%), skin (10%), fat-bodies (7%).

The total case load of 27 cases was subdivided into 52% female, 24% male, and 14% unknown.

The selected cases covered a period of 13 years and were all submitted from Northern Italy.

Table 12 shows all the main histological lesions detected in the selected retrospective samples. Lesion severity is ranged from 0 to 5, the table legend is reported below:

LESION SEVERITY:

0. No lesion
1. Mild
2. Moderate
3. Moderate to Severe
4. Severe
5. Most severe

LESION DISTRIBUTION:

0. No lesion
1. Focal
2. Multifocal (<5 lesions)
3. Multifocal (≥5 lesions)
4. Scattered
5. Diffuse

ORGAN SYSTEM	MEAN SEVERITY	MEAN DISTRIBUTION	MEAN PREVALENCE
GENITAL SYSTEM	*0*	*0*	*0%*
HEART	*0*	*0*	*0%*
SKIN	*2*	*1*	*8%*
ESOPHAGUS	*3*	*2*	*8%*
LIVER	*1*	*5*	*20%*
TONGUE AND PHARINX	*2.5*	*2*	*12%*
SPLEEN	*1*	*1*	*8%*
KIDNEYS	*4*	*5*	*8%*
STOMACH AND INTESTINE	*2*	*5*	*48%*
TRACHEA AND LUNGS	*3*	*2.4*	*25%*
URINARY BLADDER	*1*	*1*	*12%*

Table 12: Severity and distribution of histological lesions in retrospective tortoise samples.

Selected digital archive samples and the related causes of death are reported in Table 13. Amongst the 28 cases selected from the digital archive, histology blocks were only available for 22 samples (Table 13). The six missing cases were all surgical samples, ranging between 2mm and 3mm, which were completely consumed during the diagnostic process.
PCR results on the final 22 samples are recorded in Table 14.

CASE #	CAUSE OF DEATH	TORTOISE SPECIES	CASE #	CAUSE OF DEATH	TORTOISE SPECIES
EX 34/12	SUSPECT HERPESVIRUS	*T. hermanni*	EX 13/08	HERPESVIRUS ASSOCIATED INTRNUCLEAR INCLUSIONS	*T. horsfieldi*
EX 33/12	SUSPECT HERPESVIRUS	*T. horsfieldi*	EX 67/05	NECROTIZING EPATITIS	*Kinixys spp.*
EX 23/12	SUSPECT HERPESVIRUS	*T. hermanni*	EX 221/04	HETEROPHILIC TRACHEITIS AND PNEUMONIA	*Gerochelone radiata*
EX 6/12	SUSPECT HERPESVIRUS	*T. hermanni*	EX 17/04	GRANULOMATOUS HEPATITIS	*Trachemis scripta*
EX 179/10	SEPTICEMIA AND FUNGAL IPHAE	*Trachemys scripta*	EX 184/02	GRANULOMATOUS HEPATITIS	*Trachemis scripta*
EX 169/10	BACTERIAL SEPTICEMIA	*T. horsfieldi*	EX 137/02	HEPATIC DEGENERATION	*Trachemis scripta*
EX 156/10	HETROPHILIC TRACHEITIS AND NECROTIZING ENTERITIS (AMOEBA)	*T. graeca*	EX 27/02	HEPATIC DEGENERATION	*Geochelone enlongata*
EX 66/10	HERPESVIRUS ASSOCIATED INTRNUCLEAR INCLUSIONS	*T. hermanni*	EX 12/00	LYMPHOMATOUS LEUKEMIA	*Gerochelone pardalis*
EX 137/08	SUSPECT HERPESVIRUS	*T. hermanni*	EX 32/00	HETEROPHILIC RINITIS	*T. hermanni*
EX 122/08	SUSPECT HERPESVIRUS	*T. hermanni*	EX 43/00	ULCERATIVE DERMATIS	*Tryaxis spp.*
EX 96/08	SUSPECT HERPESVIRUS	*T. hermanni*	EX 45/00	HETEROPHILIC HEPATIITS	*T. elegans*

Table 13: Retrospective cases selected from the archive of the section of Veterinary Pathology of the University of Milan.

	CASE#	TeHV3 PCR results	Tortoise species
01	EX 6/12	Negative	*T. hermanni*
02	EX 33/12	Negative	*T. horsfieldi*
03	EX 34/12	Positive	*T. hermanni*
04	EX 23/12	Negative	*T. hermanni*
05	EX 179/10	Negative	*Trachemys scripta*
06	EX 169/10	Negative	*T. horsfieldi*
07	EX 156/10	Negative	*T. graeca*
08	EX 66/10	Negative	*T. hermanni*
09	EX 137/08	Positive	*T. hermanni*
10	EX 122/08	Negative	*T. hermanni*
11	EX 96/08	Negative	*T. hermanni*
12	EX 13/08	Positive	*T. horsfieldi*
13	EX 67/05	Negative	*Kinixys spp.*
14	EX 221/04	Negative	*Geocheolone sulcata*
15	EX 17/04	Negative	*Trachemys scripta*
16	EX 27/02	Negative	*Geocheolone enlongata*
17	EX 137/02	Negative	*Trachemys scripta*
18	EX 184/02	Positive	*Trachemys scripta*
19	EX 12/00	Negative	*Geochelone pardalis*
20	EX 32/00	Negative	*Testudo hermanni*
21	EX 43/00	Positive	*Tryaxis spp.*
22	EX 45/00	Negative	*Geochelone elegans*

Table 14: TeHV3 PCR results for the 22 selected retrospective samples.

Comparative evaluation of TeHV3 anatomical pathology and histopathological lesion profiles: This work aimed to investigate the lesion profile induced by TeHV3 in three distinct populations:

- A retrospective population from the anatomic pathology archive of the University of Milan
- The population from Vanzago's Oasis
- A population from diagnostic routine necropsies

To better complement the lesion profile with PCR positivity, only PCR positive cases were included in the evaluation.

Macroscopic lesions:

Macroscopic evaluation was carried out on one retrospective animal (only one animal with full necropsy was PCR positive), on all Vanzago's but two autolytic animals, and on all routine cases. All the lesions were evaluated only on PCR positive cases.

Table 15 summarizes classically TeHV3 associated gross lesions' distribution in PCR positive animals of the three examined populations. Fig. 1 to 3 show diphero-necrotic stomatitis and glossitis, nasal discharge and liver granulomas. In addition, granulomatous hepatitis (Fig. 4), massive accumulation of urates (Fig. 5) in the urinary bladder (compatible with dehydration) and suppurative and fibrinous arthritis (Fig. 6) were detected in some PCR animals. Accumulations of urates were mostly detected in very young tortoises, most of which still retained the vitelline sac residue (Fig. 5).

LESION	Vanzago Oasi	Routine cases	Retrospective samples
ULCERATIVE STOMATITIS/GLOSSITIS	13%	50%	75%
DIPHTHERIC PLAQUES	7%	0%	30%
OCULAR LESIONS	13%	0%	0%
LIVER FATTY DEGENERATION	13%	0%	100%
SEROUS ATROPHY OF THE FAT-BODIES	13%	0%	0%

Table 15: Prevalence of gross TeHV3 associated lesions in PCR positive samples.

Microscopic lesions:

The histological lesions in our case load were distributed as follows:

1) TeHVs associated inclusion bodies: Intranuclear inclusions (Figure 7) were detected in two retrospective samples and two Vanzago samples; no inclusion bodies were detected in the routine diagnostic service samples.

In Vanzago's samples, inclusion bodies were found most frequently in the tongue and trachea. In the two retrospective samples, inclusion bodies were most commonly detected in the lungs. Occasionally, cytopathic effects (e.g. Syncytia – Figure 8) were also detected in PCR positive animals.

2) Neoplasms: Only one retrospective sample was diagnosed with a tumor. The subject was affected by a lymphomatous leukemia (Figure 9-11)

3) Necrotic lesions: were found to be dispersed, but the most affected organs were the liver (44% of animals), large intestine (31% of animals), tongue (25% of animals) and esophagus (25% of animals).

4) Bacteria: The most affected organs were the small and large intestine (31% of subjects) and liver (31% of subjects). In 57% of these subjects there were also bacterial embolisms of various organs (especially lungs and kidneys) associated with host inflammatory responses, which suggests *ante-mortem* bacterial proliferation. In most cases the bacteria population consisted of bacilli, and where a mixed bacteria population was present, bacilli were frequently the most relevant component (Figure 12-14).

5) Mycosis: Fungal hyphae were only detected in two subjects. One subject was characterized by the presence of an ulcerative dermatitis, the other by an extensive ulcerative glossitis.

6) Inflammation: Seventy percent of the animals showed randomly distributed inflammatory processes. The most common inflammatory process type was heterophilic (88% of subjects) (Fig. 11-12) while granulomatous (Figure 15-16) and lymphocytic inflammation were detected in a smaller number of cases (46% of the animals). Among the examined cases, a very low percentage of mixed inflammatory processes were detected (13%). Rhinitis was only detected in one retrospective case (EX 32/00).

Heterophilic inflammation was mainly detected in the large intestine (41% of the subjects), small intestine (33% of the subjects), liver, kidneys and lungs (31% of the subjects).

Granulomatous lesions were mainly found in the liver (Fig. 3), less frequently in the lungs and kidneys.

7) Extramedullary hematopoiesis: was detected in the spleen (50% of animals), liver (40% of animals), and kidneys (about 10% of animals) of five tortoises (Figure 17).

Regarding the severity of this finding, most of the cases were mild forms and more rarely intermediate forms. Mild cases were characterized by erythropoiesis only. Moderate cases might be marked by either erythropoiesis and leukopoiesis, or by the presence of erythropoiesis in multiple organs.

7) Edema: the most affected organs were the lungs (56% of subjects), small and large intestine (33% of subjects respectively), liver, urinary bladder and trachea (11% of subjects respectively). Most of the cases were post mortal changes, with no other associated lesions.

8) Ulcerative and erosive lesions: the most affected organ was the tongue (50% of subjects) followed by the small and large intestine (33% of subjects), esophagus and trachea (25% of subjects).

Ulcers and erosions were also detected in the trachea and esophagus of one subject.

In two of the tortoises mentioned above, intestinal ulcerations involved the serosa inducing a severe coelomitis.

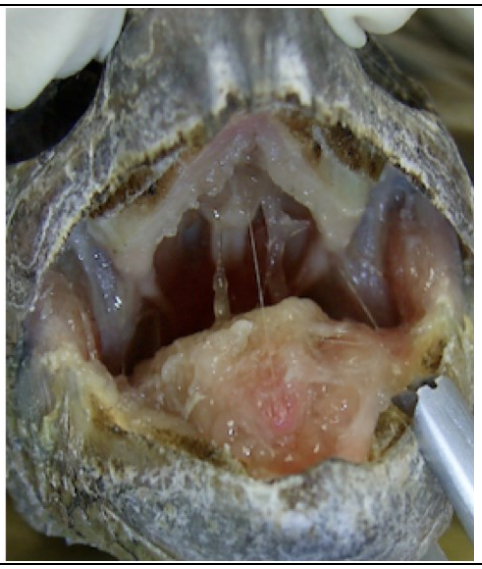
9) Thrombosis: only one tortoise was found to have *intra vitam* thrombosis, with thrombi formation at the level of the liver, heart, lungs, and pancreas. Some of the examined thrombi were also associated with intravascular bacteria, suggesting a disseminated intravascular coagulation (D.I.C.) secondary to a septicemic disease.
10) Hemorrhages: the largest number was detected in the lungs (33% of subjects) (Figure 18), while the stomach, small and large intestine, urinary bladder and trachea were less affected (17% of subjects each). Fifty percent of the detected hemorrhages were localized, followed by multifocal (35% of subjects) and diffuse forms (15% of subjects).
11) Fibrin: approximately 30% of animals had fibrin deposits. The tongue was the most common site of deposition (44% of subjects), followed by the liver (33% of subjects), small and large intestine and urinary bladder (11%).
12) Abscesses: multifocal abscesses were observed at the level of the liver, spleen, kidneys, and lungs of one subject. No other abscesses were identified.
13) Mineralization: was detected in two subjects. The most affected organ was the kidney (50% of subjects), followed by the small and big intestine, skin, and pancreas (25% each). Mineralization was always multifocal.
14) Metabolic diseases: the most common metabolic change was the presence of a "fatty liver," histologically cases associated with both lipidosis and glycogenosis (Figure 19) were detected. Fat-body atrophy was the second most common finding.
15) Gout: visceral gout was detected in the 30% of the examined tortoises. Articular gout was only detected in one subject.
TeHV3 related histological lesions were evaluated on five retrospective samples (EX 34/12, EX 13/08, EX 137/08, EX 184/02, EX 43/00), five Vanzago samples (PC 184/14, PC 191/14 and PC 193/14 were excluded because of the presence of severe autolytic changes), and four routine diagnostic samples (EX 116/14, EX 90/15, EX 114/15, EX 24/17). All lesions were only evaluated on PCR positive cases.
16) Parasites: they were found mainly at the gastric (33% of the animals) and the large intestine (67% of the animals) level.
Most subjects were infested by *Ossiurid* spp. (Figure 20) in adults having been exposed to ciliated protozoa such as *Balandium* spp. and *Nyctotherus* spp., only one subject had a serious form of infestation by amoeba (probably *Entamoeba histolitica*) and a subject featured a single myocardial parasitic cyst from *Sarcocystis* spp.

Table 16 reports the prevalence of classically associated TeHV3 lesions in our caseload.

LESION	Vanzago Oasi	Routine cases	Retrospective samples
NECROTIZING GLOSSITIS	14.3%	50%	33.3%
NECROTIZING TRACHEO-BRONCHITIS	25%	25%	0%
PNEUMONIA	43%	25%	0%
HEPATITIS	0%	0%	44.4%
GRANULOMATOUS HEPATI-TIS	28.6%	0%	22.2%
GRANULOMATOUS PNEUMO-NIA	14.3%	0%	11.1%
INTRANUCLEAR INCLUSION BODIES	28.6%	0%	33.3%
HETEROPHILIC RHINITIS	0%	0%	11.1%

Table 16: Prevalence of classically TeHV3 microscopic lesions in our PCR positive caseload.

GROSS AND HISTOLOGICAL LESIONS IDENTIFIED IN RETROSPECTIVE AND PROSPECTIVE TURTLES INCLUDED IN THIS STUDY



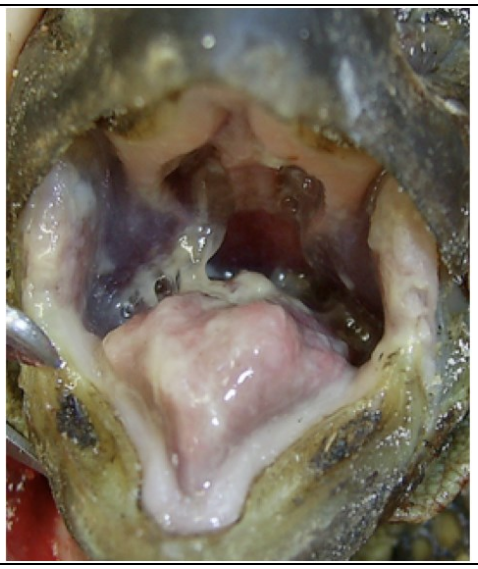


Figure 1: Diphtero-necrotic glossitis and stomatitis (TeHV3 associated)

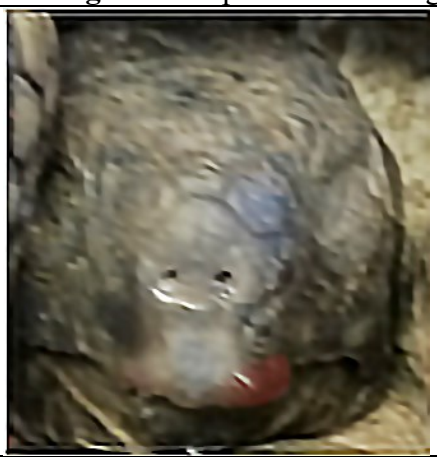


Figure 2: Mucopurulent rhinitis

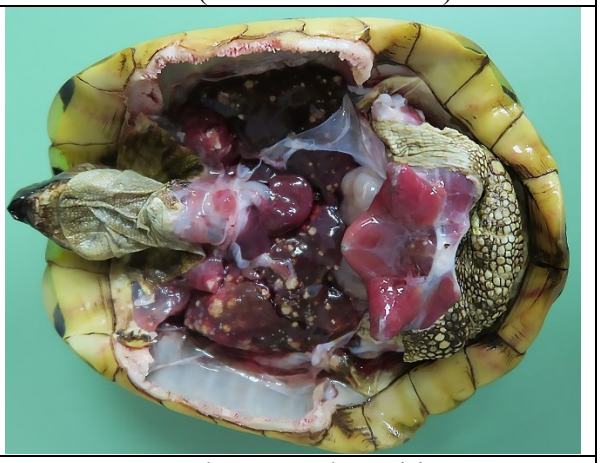


Figure 3: Granulomatous hepatitis

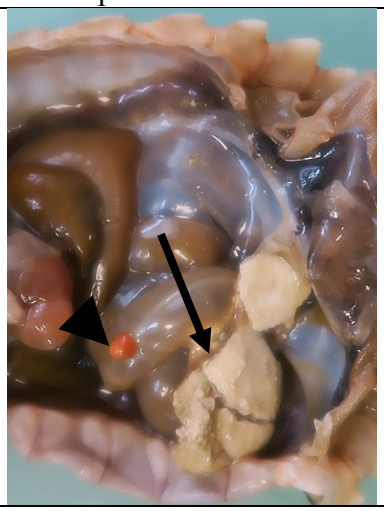


Figure 5: Urate aggregates in the urinary bladder (arrow) in a very young tortoise (*T. hermanni*) still presentig residual yolk site (arrow head).

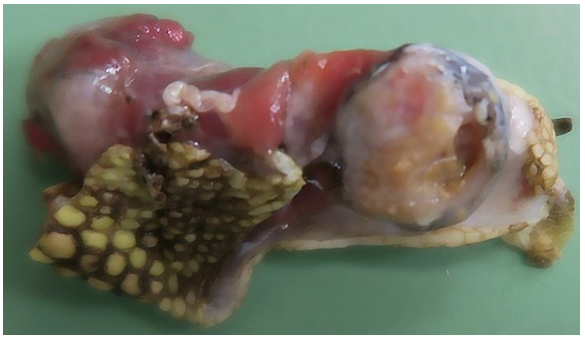


Figure 6: Bacterial osteoartritis, *T. graeca*.

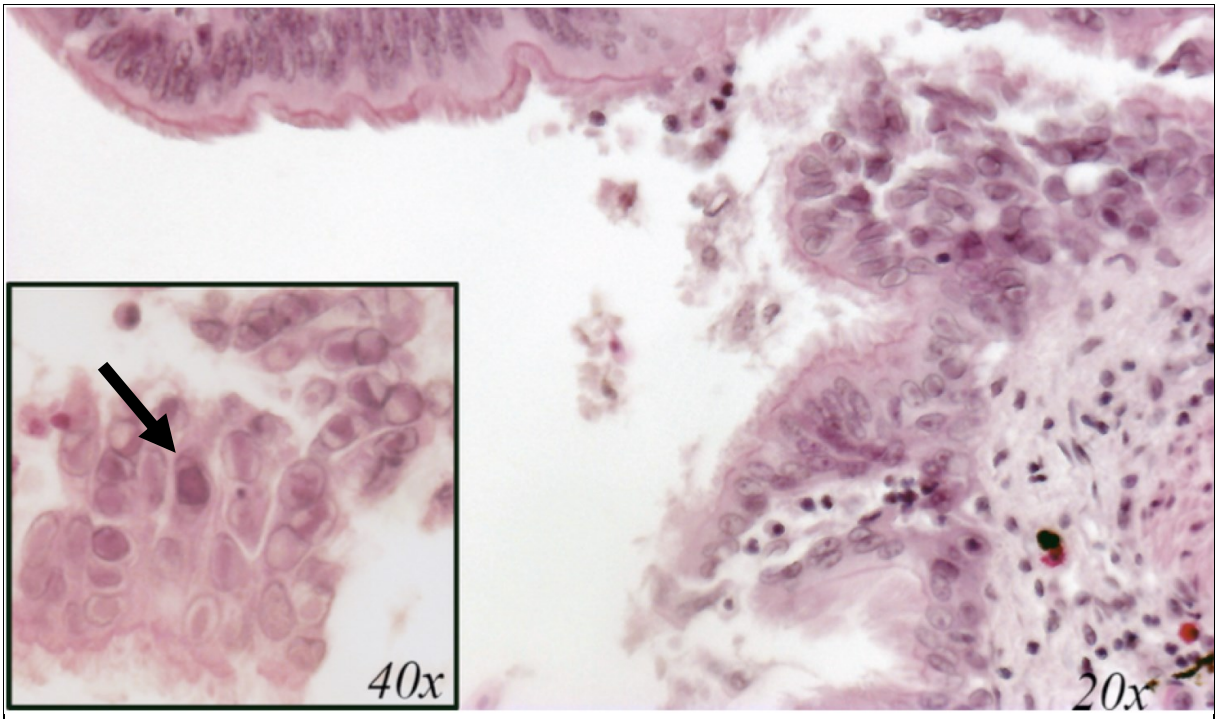


Figure 7: TeHV1 associated inclusion bodies and heterophilic pneumonia in a *T. horsfieldi* - H&E – 20 x
Inset: Detail of the amphophilic intranuclear inclusion bodies – H&E - 40 x

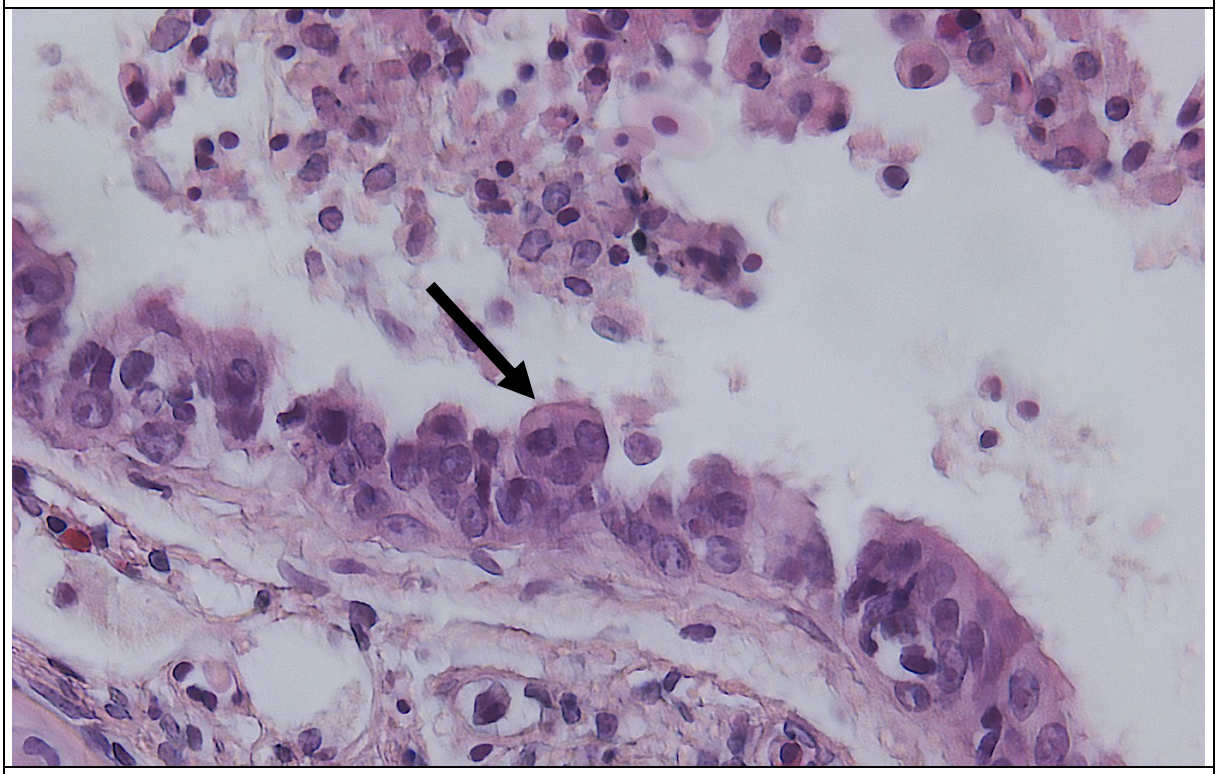


Figure 8: Trachea –Multinucleated epithelial cell (syncytia) in a TeHV3 PRC positive tortoise (arrow). No inclusions were detected in this animal – H&E – 40x

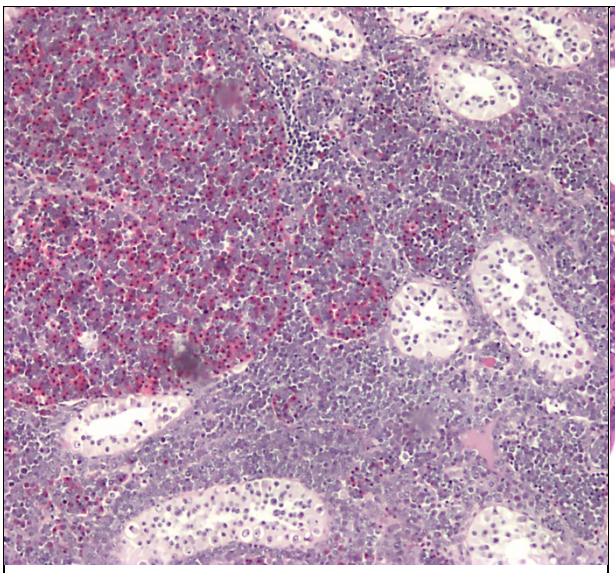


Figure 9: Kidney – Medium cell lymphoma, H&E – 1.6x

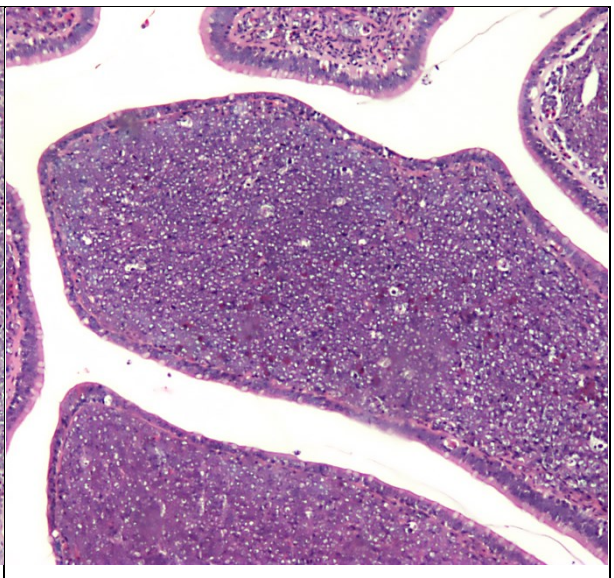


Figure 10: Small intestine – Medium cell lymphoma – H&E - 5x

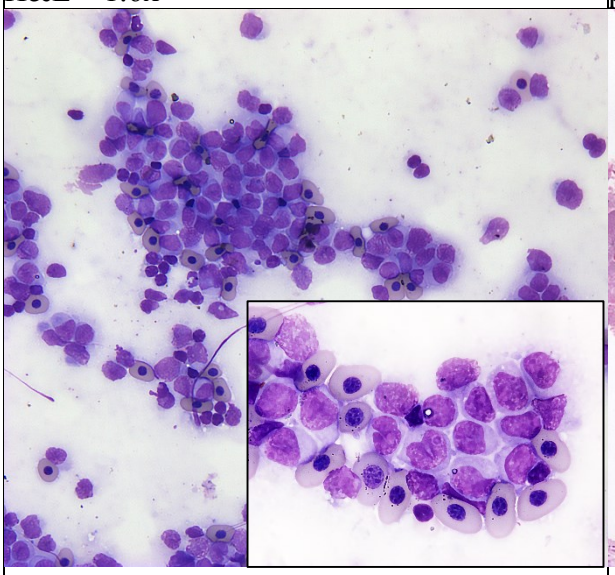


Figure 11: Imprint cytology from the spleen – Medium cell lymphoma – May-Grünwald-Giemsa - 10x
Insert: Same cell population - May-grünwald-Giemsa - 40x

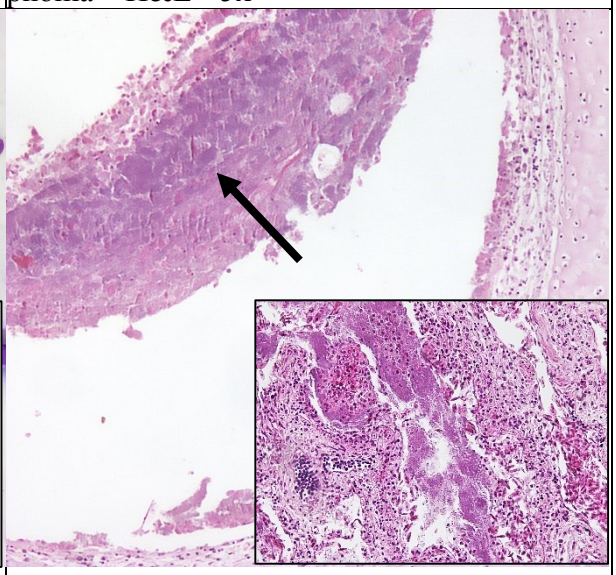


Figure 12: Trachea – Necrotizing and heterophilic tracheatis with intralesional bacterial aggregates (arrow) – H&E - 5x
Insert: High power of the bacterial aggregates also present in the lungs– H&E - 10x

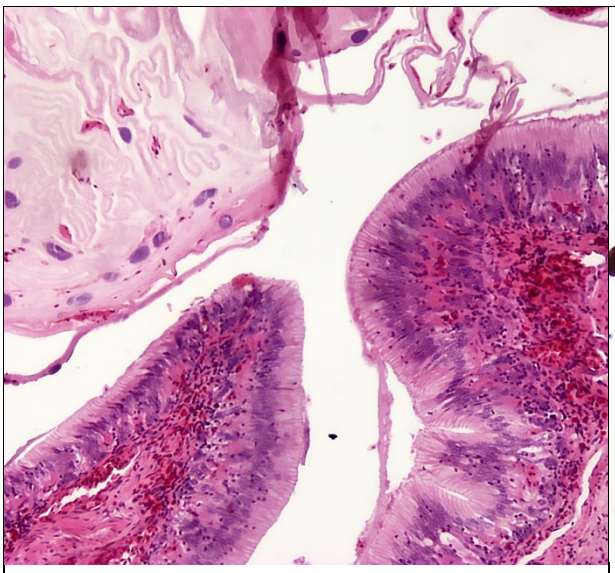


Figure 13: Small intestine – Heterophilic and catarrhal enteritis, with abundant intraluminal mucous admixed with bacteria – H&E - 5x

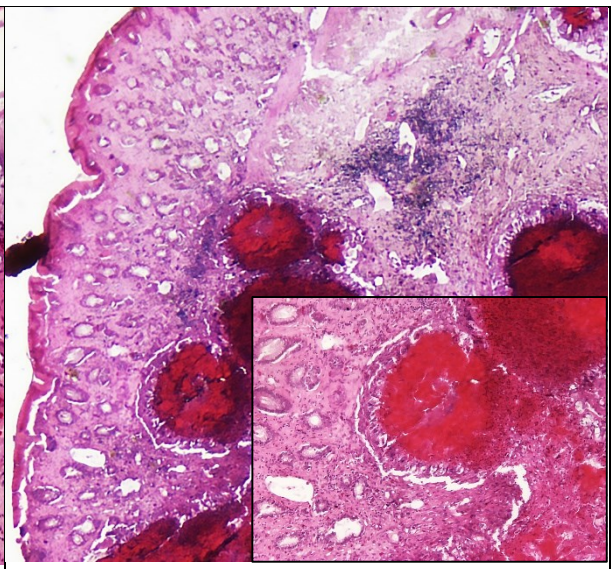


Figure 14: Large intestine – Heterophilic granulomas in the submucosa – H&M – 1.6x
Insert: High power of the granulomas– H&E - 10x

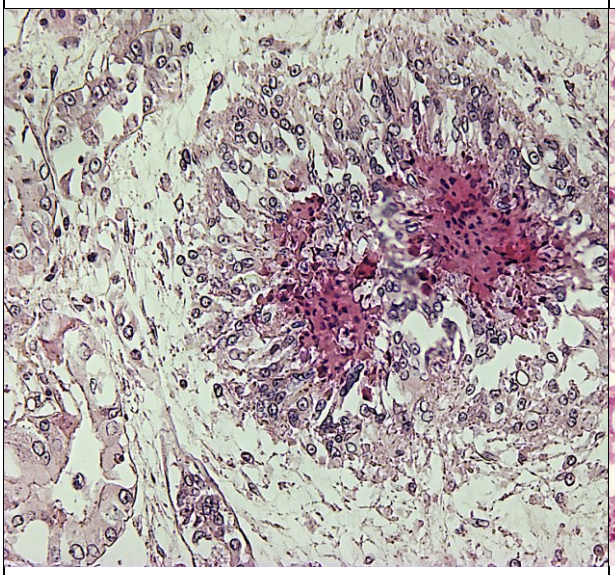


Figure 15: Kidney – Heterophilic granulomas in the renal interstium, centers on optically cleat needle-shaped crystals compatible with urate crystals (gout) – H&E – 10x

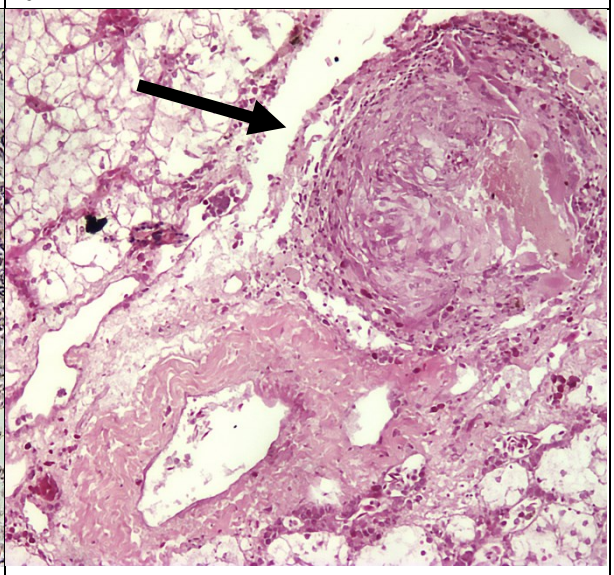


Figure 16: Fat bodies – Histiocytic granuloma (arrow) – H&E – 5x

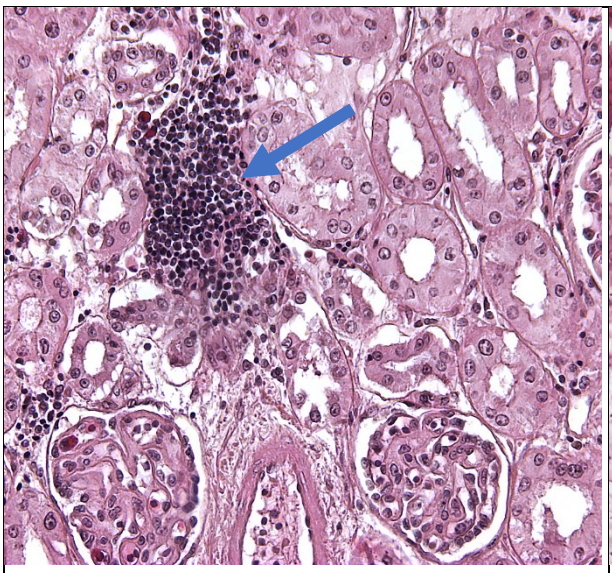


Figure 17: Kidney – EMH (arrow) – H&E – 10x

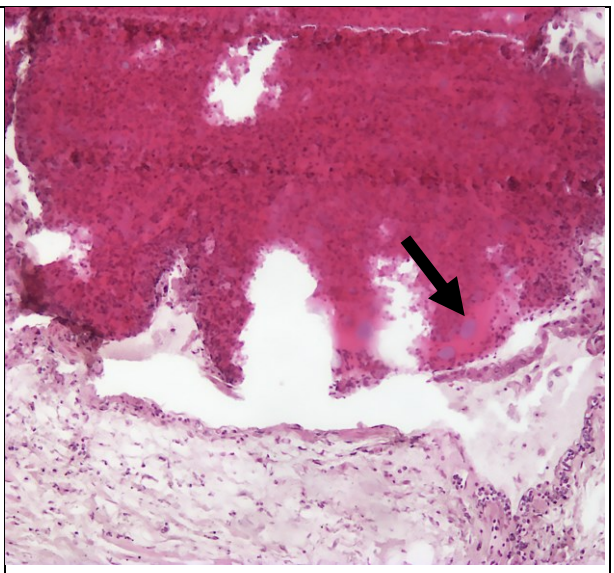


Figure 18: Lung – Extensive faveolar and aeducular hemorrhages with bacteria (arrow) – H&E – 10x

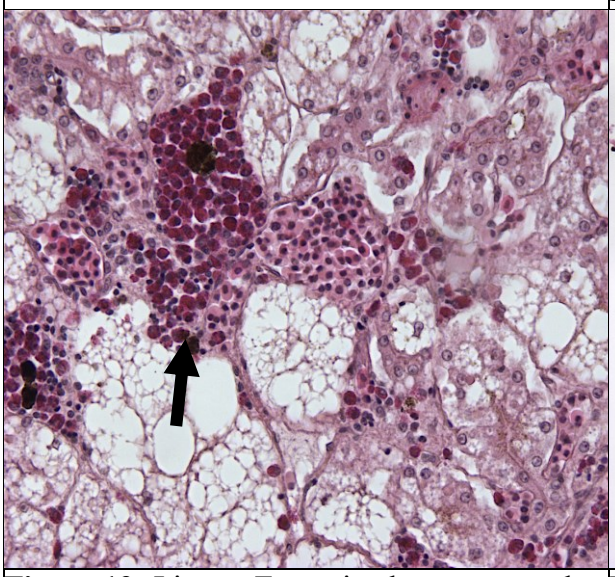


Figure 19: Liver – Extensive hepatocytes glycogenosis with heterophilic hepatitis (arrow) – H&E – 5x

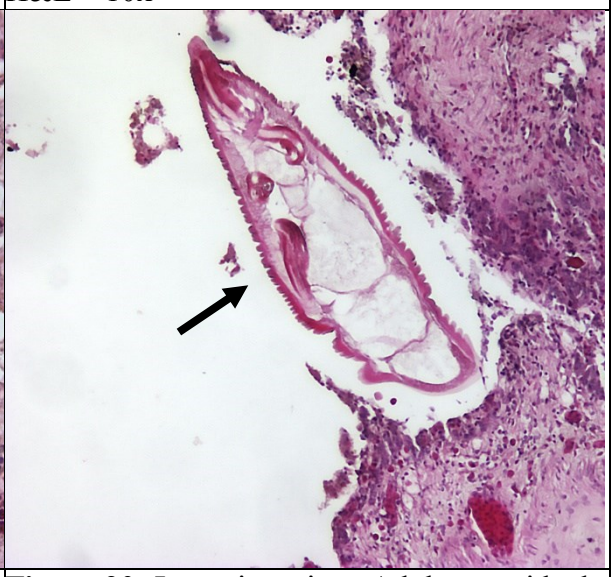


Figure 20: Large intestine –Adult oxyurid adherent to intestinal mucosa (arrow) – H&E – 5x

PCR on retrospective samples:

Five samples returned as PCR positive, among these four were TeHV3 positive and one TeHV1 positive (EX 13/08).

Viral isolation:

Among all Vanzago and routine cases, viral isolation was only successful from one tortoise (PC 192/14).

Early cytopathic effects, including syncytia formation and single cell necrosis were detectable 30 days after the infection.

Discussion:

The aims of this work were to:

- Identify the presence of TeHVs in Italy.

- Compare TeHVs' lesion profile documented in literature with the one identified in our case-load.

Identification of TeHVs in Italy:

Although tested for the presence of TeHV1, TeHV2, and TeHV4, all our samples were only TeHV3 positive, but one retrospective case was positive for TeHV1 (EX 13/08).

TeHV3 prevalence:

The prevalence in our study was:

- Retrospective samples: 25% (five PCR positive case out of 20 selected)

- Vanzago's Oasis: in this case two different considerations have to be made. ELISA test positive animals were 16 out of 17, for a total seroprevalence of 94.12%. PCR positive tortoises were 8 out of 18 for an overall prevalence of 44.4%.

- Routine diagnostic cases: four out of ten were PCR positive, for a prevalence of 40%.

Molecular evaluation of our case load demonstrates that TeHV3 is diffusely present in Northern Italy, and the first cases can be dated back to 2000 (retrospective case EX 43/00). This result traces the presence of this virus on Italian soil before the very first report of it by Origgi FC [8] in 2003.

Only few published works worldwide report the presence of TeHVs. The prevalence of TeHV2 has been investigated in desert tortoises (*Gopherus agassizii*) by Jacobson et al. in 2012 [5] and later by Braun et al. in 2014 [9], who reported a prevalence of 31% and 48%, respectively.

The only study investigating TeHV3 prevalence in Europe was performed by Soares et al. (2004) [10], and reported a seroprevalence for TeHV3 and *Mycoplasma agassizii* of 8.2% in the UK.

Results obtained from our case load have to considered group by group.

Retrospective samples:

This group contained samples from 1999 to 2012. The overall prevalence of TeHV3 in these samples is 25%, which is very close to the data (31%) obtained by Jacobson, although it is higher than the previously reported prevalence of TeHV3 evaluated by Soares.[10]

The gap between our and Soares' results might be explained in different ways. Soares investigated the presence of TeHV3 between 1999 and 2002. Among our retrospective cases, only seven fit in that time range. If only those samples are considered, two positive cases out of seven, the overall prevalence remain 25%. During the 90s, the most common pet tortoise in United Kingdom (UK) was *Terrapene* spp., which is susceptible to different herpesviruses that might not be amplified by Soares probes. Furthermore, it is also possible that the overall prevalence of TeHV3 in UK was lower in that specific time range.

The Vanzago Oasis samples:

Two different prevalences were calculated for this population:

- Seroprevalence: 94.12%
- PCR: 44.4%

Vanzago's population was living in a small enclosure, where animals were introduced without testing and quarantine, but only after clinical examination. Animals in close contact might have come in contact with the virus, developing antibodies and resulting seropositive. However, the PCR based prevalence was much lower, suggesting that the virus was present in less than half of the animals. Unfortunately, no introduction records were available, and we could not establish when the animals were introduced to the Oasis. Our positive sample prevalence was calculated by dividing the number of positive animals by the total number of examined tortoises (18) and multiplying that number by 100.

Prevalence grouped by tortoise species was:

- Four *Testudo hermanni* (out of seven) for a prevalence of 57.14%
- One *Testudo graeca* (out of two) for a prevalence of 5.8%
- Two *Testudo marginata* (out of nine) for a prevalence of 22%.

Although the distribution of our population was not homogeneous, our data support literature, that states that *T. hermanni* and *T. marginata* seem more susceptible to TeHV3 infections compared to *T. graeca*.

The different susceptibility of tortoises to TeHV3 has not yet been explained, although two distinct theories can support this difference:

- Coevolution with the virus: the most diffuse explanation suggests that the virus evolved with *T. graeca*.
- Tortoises' evolution: Van der Kuyl et al. (2002) [11] demonstrated that, based on the 12S rRNA mitochondrial subunit gene sequence, two different taxa are present in the clade *Testudo* spp.

One taxon includes *T. margianta*, *T. graeca* e *T. kleinmanni*. The other taxon includes *T. hermanni* and *T. horsfieldi*.

Probably both theories are correct. It is indeed possible that the virus started evolving in an ancestor of *T. marginata* and *T. graeca*, causing the immune system of these tortoises to be more efficient against this virus; this is further supported by the presence of a single report describing a herpesvirus infection in *T. kleinmanni*.

Prospective cases:

PCR-based prevalence in our routine samples was 40% (four positives out of ten). Despite the limited number of cases, the PCR-based prevalence in the routine cases was close to the one calculated via PCR in Vanzago's Oasis and to the prevalence estimated for TeHV2 in the California desert, suggesting that TeHVs prevalence is increasing worldwide.

Macroscopic and microscopic lesion profiles:

Stomatitis and glossitis associated with the presence of diphtheronecrotic plaques are reported in the literature as "pathognomonic" lesions. Stomatitis and glossitis were macroscopically detected in 13%, 50%, and 75% of Vanzago, routine, and retrospective cases respectively. Among these cases, diphtheric plaques were detected in 7%, 0%, and 30% respectively of the Vanzago, routine, and retrospective cases. Although the mean incidence of stomatitis and glossitis is 46%, which is in agreement with literature, the variability among the samples is high. Furthermore, the diphtheric plaques' mean value is 12.3%, which is lower than the incidence reported in literature. As mentioned in the "Results section" some of the examined cases, especially those from Vanzago's Oasis, were characterized by post mortal changes not allowing to properly evaluate lesions from both a macroscopic and microscopic point of view. Furthermore, two tortoises with lesion profiles strongly suggestive of a herpesvirus infection (EX 66/10 and EX 34/12) were PCR negative. Although the case EX 66/10 showed the presence of intranuclear amphophilic inclusion bodies, the case was repeatedly negative for different herpesvirus PCR, including Pan herpes, TeHV1, TeHV2, TeHV3, and TeHV4. These two examples demonstrate that overinterpretation might occur when only based on microscopic

and gross findings. According to literature, one of the most important lesions induced by TeHV3 is heterophilic hepatitis, but in our study only nine animals showed this injury (44%, four out of 20) and only among the retrospective samples. [4, 12] The presence of heterophilic hepatitis in only four animals out of 50 (22 retrospective, 18 from Vanzago and 10 from routine cases) does not suggest this might be a true significant lesion.

According to Mader et al. (2006) [13], granulomatous lesions of the liver are associated with TeHVs infection, and this is coherent with what we found (28.6% Vanzago's Oasis, 22.2% retrospective samples), although no granulomatous hepatitis was detected in the routine diagnostic specimens.

Heterophilic rhinitis has been reported by Mader (2006) [13], as one of the most common TeHVs lesions. Heterophilic rhinitis was detected in two retrospective samples (one *T. heremanni* and one *T. horsfieldi*), from a PCR negative tortoise. PCR negativity suggests that these lesions might be correlating with other pathologic processes than TeHVs.

Intranuclear inclusion bodies were detected in 28.6% of Vanzago's and 33.3% of the retrospective samples, while no inclusions were detected in the routine cases. Origgi et al. [14] reported that inclusion bodies are viable for four weeks after infection, which might justify why only a few cases were identified. The distribution identified in this study is lower than what is reported in literature.

The abovementioned findings suggest that histopathology should be limited to post-mortem examinations. [14] Although some lesion profiles are reported as "pathognomonic" [14], the concept of "pathognomonic" should be deprecated in modern pathology, because many lesion profiles of different diseases may overlap and atypical presentations of typical conditions are not infrequent, leading to misdiagnoses. Despite the presence of "pathognomonic" lesions in our caseload, our findings suggest that viral identification should always be carried out to confirm the actual presence of the virus and exclude other possible causes. The latter support the use of more precise terms such as "extremely relevant" or "commonly associated with" that are progressively replacing the word "pathognomonic" in pathology.

According to our findings, there are two main limiting factors in TeHVs' anatomical pathology interpretation:

- Conservation status of the entire body or body parts

- Confounding factors
- Pathologist experience

One of the most relevant aspects we identified in our samples is that the cause of death of most of the animals was related to secondary infection more than to the TeHVs lesions themselves. Prevention of these secondary injuries might have a pivotal role in preventing subject death, reducing the mortality associated with TeHVs.

Genomic data (see chapter TeHV3 genome), suggest that TeHV3 might be responsible for host immune system modulation; this finding in combination with the seasonal immune suppression tortoises physiologically experience during the winter might be responsible for the onset of the secondary infections we observed.

We also detected the very first case of TeHV1 reported in Italy (EX 13/08); this was detected in a *T. horsfieldi*. Most of the cases of TeHV1 are reported in *T. horsfieldi*, which suggests that this tortoise species might be the target of the virus.

The neoplastic lesions we selected from the archive were negative for TeHVs, suggesting that TeHV3 is not involved in neoplastic disease of tortoises. However, this hypothesis needs to be confirmed by using a higher case load.

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TESTUDO NECROPSY DATA COLLECTION WALKTHROUGH

Necropsy guide to simples collection in Testudo spp.

*Required

1. **NECROPSY PROTOCOL** *
   Database ref. number

2. **TESTUDO SPECIES IDENTIFICATION** *
   Define species
   Tick all that apply.

   - [ ] Testudo graeca graeca
   - [ ] Testudo graeca iberia
   - [ ] Testudo graeca terrestris
   - [ ] Testudo graeca zarudnyi
   - [ ] Testudo graeca flavominimaralis
   - [ ] Testudo graeca whitei
   - [ ] Testudo graeca nabulensis
   - [ ] Testudo hermanni hermanni (robertmertensi)
   - [ ] Testudo hermanni boettgeri
   - [ ] Testudo hermanni (Agrionemys) horsfieldi
   - [ ] Testudo marginata
   - [ ] Testudo kleinmanni
   - [ ] Other

3. **FILL IF OTHER**

4. **GENDER IDENTIFICATION**
   Sex identification
   Tick all that apply.

   - [ ] Male
   - [ ] Female

5. **LENGHT**
   From head to the caudal part of the dorsal scutum

6. **WIDTH**
   From side to side of the scutum

7. **PROVENANCE**
   Turtle provenance

8. **BODYWEIGHT**

9. **LIFE CONDITION**
   Where the tortoise live?
   Tick all that apply.

   - [ ] Wild
   - [ ] Domestic
   - [ ] Half-wild

10. **ORAL DABBER**
    For cellular colture
    Tick all that apply.

    - [ ] Done

11. **ORGANS SPECIMENS FOR PCR**

2 simple for any organ one in RNA-later and one in empty cuvette
Tick all that apply.

- [ ] Brain - A (sempre)
- [ ] Tongue, Farinx - B
- [ ] Trachea - C
- [ ] Esophagus - D
- [ ] Liver - E
- [ ] Lungs - F
- [ ] Stomach, Gut - G
- [ ] Genital tract - H
- [ ] Kidney - I
- [ ] Heart - L
- [ ] Gum (if damaged) - M

12. **ORGANS SPECIMENS FOR HISTOLOGY**

1 simple for any organ
Tick all that apply.

- [ ] Brain (no if frozen)
- [ ] Tongue, Farinx
- [ ] Trachea
- [ ] Esophagus
- [ ] Liver
- [ ] Lungs
- [ ] Stomach, Gut
- [ ] Genital tract
- [ ] Kidney
- [ ] Heart
- [ ] Eye (if damaged)
- [ ] Gum (if damage)
- [ ] Pancreas
- [ ] Spleen
- [ ] Turtle shell

13. **ORGANS SPECIMENS FOR CYTOLOGY (NO IF FROZEN)**

1 simple for any organ, apposition
Tick all that apply.

- [ ] Brain
- [ ] Tongue, Farinx
- [ ] Trachea
- [ ] Esophagus
- [ ] Liver
- [ ] Lungs
- [ ] Stomach, Gut
- [ ] Genital tracts
- [ ] Gum scrub
- [ ] Kidney
- [ ] Heart

14. **BLOOD SMEAR (NO IF FROZEN)**

1 simple for animal
Tick all that apply.

- [ ] Done

15. **LESIONS SEVERITY [TONGUE AND FARINX]**

Mark only one oval per row.

	1	2	3	4	5
TONGUE AND FARINX					
TRACHEA					
ESOPHAGUS					
LIVER					
LUNGS					
STOMACH AND GUT					
GENITAL TRACT					
KIDNEY					
GUM					
HEART					
SKIN					
TORTOISE SHELL					
PLASTRON					

16. **LESIONS DISTRIBUTION [TONGUE AND FARINX]**
Mark only one oval per row.

	1	2	3	4	5
TONGUE AND FARINX					
TRACHEA					
ESOPHAGUS					
LIVER					
LUNGS					
STOMACH AND GUT					
GENITAL TRACT					
KIDNEY					
GUM					
HEART					
SKIN					
TORTOISE SHELL					
PLASTRON					

17. **LESIONS OF G.I. TRACT**
Tick all that apply.

- [ ] ULCERATIVE STOMATIS
- [ ] ULCERATIVE GLOSSITIS
- [ ] ULCERATIVE LESIONS OF G.I. MUCOSA
- [ ] ORAL DIPHTERIC PLACQUES
- [ ] NECROTIZING STOMATITIS
- [ ] NECROTIZING GLOSSITIS
- [ ] ENTERITIS

18. **LESIONS OF RESPIRATORY TRACT**
Tick all that apply.

- [ ] NECROTIZING TRACHEITIS WITH CASEUS EXUDATE
- [ ] NECROTIZING BRONCHITIS
- [ ] PNEUMONIA WITH ENPHYSEMA
- [ ] SEVERE BRONCOPNEUMONIA
- [ ] ULCERATIVE LESIONS OF RESPIRATORY TRACT

19. **LESIONS OF THE LIVER**
Tick all that apply.

- [ ] HEPATOMEGALY
- [ ] HEPATITIS
- [ ] FATTY DEGENERATION

20. **LESIONS OF THE FAT BODIES**
Tick all that apply.

- [ ] SERUS ATROPHY

21. **LESION OF EYE**
Tick all that apply.

- [ ] EYE LESIONS

22. **LESIONS OF THE SKIN**
Tick all that apply.

- [ ] PAPULAR LESIONS
- [ ] ULCERATIVE LESIONS (skin - plastron - turtle shell)
- [ ] TURTLE PLASTRON BUCKLING

23. **NOTES ON SKIN LESIONS**

24. **LESION OF SPLEEN**
Tick all that apply.

- [ ] SPLENIOMEGALY
- [ ] LYMPHOID DEPLETION (during dormacy is normal)

25. **FIBROPAPILLOMAS**
Tick all that apply.

- [ ] SKIN
- [ ] LUNGS
- [ ] KIDNEY
- [ ] G.I.

26. **GRANULOMATOUS LESIONS**

Tick all that apply.

- [ ] LIVER
- [ ] SKIN
- [ ] SPLEEN
- [ ] TONGUE AND PHARINX
- [ ] LUNGS
- [ ] PANCREAS
- [ ] TRACHEA
- [ ] BRAIN
- [ ] STOMACH
- [ ] GUT
- [ ] ESOPHAGUS
- [ ] GUM
- [ ] EYE
- [ ] HEART
- [ ] KIDNEY

27. **COMMENTS**

Anything to emphasize?

Scientific communications related to the research argument:

SisVet 2015

XII congress of Italian association of Veterinary Pathologists (A.I.P. Vet)

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TeHV3 outbreak characterization in captive *Testudo* spp.

Marco Tecilla [1], Francesco Carlo Origgi [2], Guido Grilli [1], Annalisa Forlani [1], and Paola Roccabianca [1]

[1] *Facoltà di Medicina Veterinaria, Università degli Studi di Milano*

[2] Vetsuisse Faculty, University of Bern.

Tortoises represent a popular non-conventional pet in Italy. Of these, several species are either considered endangered (*Testudo hermanni*) or near threatened (*T. marginata* and *T. graeca*) according to the Italian com- mission of the International Union for Conservation of Nature. When pet tortoises are abandoned or found injured or seized following illegal detention, they are sent to wildlife rehabilitation centers. Starting from 2008, the *Testudo* spp. population housed in the WWF Vanzago's oasis exhibited clinical signs of variably severe lethargy, nasal discharge, conjunctivitis and diphtheronecrotic glossitis. During that period of time 50 tortoises died with reported mortality peaks during March and October. In Spring 2012, the Vanzago center population was composed of 9 *T. marginata*, 7 *T. hermanni* and 2 *T. graeca*, still variably exhibiting the same abovementioned clinical signs. By the end of 2012 all *Testudo* species had died. Based on these findings, *Testudinid herpesvirus* 3 (TeHV3) infection was suspected. The presence of TeHV3 was investigated by molecular biology and anatomical pathology. All the tortoises housed in Vanzago were tested for the presence of anti-TeHV3 antibodies by ELISA and they all resulted positive but one *T. hermanni*. Of these, 3 *T. marginata*, 2 *T. graeca* and 12 *T. hermanni* died and were all necropsied. Lesion frequency distribution was lingual and oral diphtheric plaques (15.4%), serous atrophy of the fat (23.1%), hepatic lipidosis (15.4%) ulcerative stomatitis and/or glossitis (7.7%), pneumonia with emphysema (43%), focal intralesional bacterial aggregates (17%), intravascular bacterial thrombi (25%), hepatic granulomas (28.6%), necrotizing tracheobronchitis (25%) and intranuclear amphophilic/eosinophilic inclusion bodies (8.33%). PCR con- firmed the presence of the virus in 8/12 tortoises. To better complement the epidemiological evaluation of TeHV3 distribution in northern Italy tortoises, 20 retrospective cases were selected from

the archive of the University of Milan. Selection criteria were the presence of inclusion bodies or necrotizing lesions of the respiratory or gastrointestinal tract. Of the 20 cases, 5 were TeHV3 PCR positive tortoises. Lesions closely resembled those of the Vanzago's population but there were more cases with diphtero-necrotic glossitis and stomatitis and inclusions bodies. These results are consistent with a high prevalence of TeHV3 in northern Italy tortoises household population. The finding of intranuclear inclusion bodies was specific but did not represent a sensitive diagnostic tool. TeHV3 diagnostic gross and microscopic lesions have been reported to vary according with the host immune response and by the viral replicative status, and can be obscured by autolytic changes, thus gross and microscopic findings are not always diagnostic and the support of additional techniques is often necessary to confirm viral infection. In the current caseload, TeHV3 infection was often associated with secondary lesions suggestive of immunodepression that can be attributed to virus itself associated with abnormal hibernation. According to the literature and to our findings, *T. hermanni* spp. seems the species with higher mortality and lower antibody concentrations when infected with TeHV3

Francesco C. Origgi (2012) *Testudinid herpesvirus* es: A Review. Journal of Herpetological Medicine and Surgery (2012), Vol. 22 (1-2), pp. 42-54

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE PER LA SALUTE, LA PRODUZIONE ANIMALE E LA SICUREZZA ALIMENTARE

CORRESPONDING AUTHOR
Marco Tecilla,
marco.tecilla@unimi.it

TeHV3 outbreak characterization in captive *Testudo spp.*

M. Tecilla[1], P. Roccabianca[1], G. Grilli [1], F. Forlani [1], F.C. Origgi [2]

[1] *Department of Veterinary Science and Public Health, Università degli Studi di Milano, Via Celoria 10, 20133 Milan, Italy*

[2] *Center for Fish and Wildlife health, Vetsuisse-Faculty of Bern, Laenggassstrasse 122, 3012 Bern, Switzerland*

ABSTRACT

Italian Tortoises species are considered either endangered or near threatened according to International Union for Conservation of Nature. When pet tortoises are abandoned or found injured or seized following illegal detention, they are sent to wildlife rehabilitation centers. From 2008, the *Testudo spp.* population housed in the WWF Vanzago's oasis exhibited clinical signs compatible with *Testudinid herpesviurs 3* (TeHV3) infection. By the end of 2012 all *Testudo* had died. The presence of TeHV3 was investigated by molecular biology and pathology. All the tortoises housed in Vanzago resulted ELISA positive for the presence of anti-TeHV3 antibodies except one *T. hermanni*. Of these, 12 animals died and were all necropsied. Lesion frequency distribution was evaluate by histology. PCR was positive in 8/12 tortoises. To better complement the epidemiological evaluation of the virus in northern Italy, 20 retrospective cases were selected from the archive of the University of Milan. Of these, 5 were TeHV3 PCR positive. Lesions closely resembled those of the Vanzago's population. These results are consistent with a high prevalence of TeHV3 in northern Italy. The finding of intranuclear inclusion bodies demonstrated to be specific but not sensitive. TeHV3 diagnostic pathological lesions have been reported to vary according with host immune response and by the viral replicative status. Molecular techniques were often necessary to confirm the infection. According to the literature and to our findings, *T. hermanni spp.* seems the species with higher mortality and lower antibody concentrations when infected with TeHV3.

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Chapter 6: The genome of *Testudinid herpesvirus* 3.

Original Title: A Genomic Approach to Unravel Host-Pathogen Interaction in Chelonians: The Example of *Testudinid herpesvirus* 3

Authors: Francesco C. Origgi, **Marco Tecilla**, Paola Pilo, Fabio Aloisio, Patricia Otten, Lisandra Aguilar-Bultet, Ursula Sattler, Paola Roccabianca, Carlos H. Romero, David C. Bloom, Elliott R. Jacobson.

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Introduction:

Herpesviruses have been reported as significant pathogens of snakes [1, 2], lizards [3–10] and alligators [11, 12], however, more herpesviruses have been detected in chelonians than in any other reptilian taxa [13]. Presently, chelonian herpesviruses have been detected and/or isolated from both turtles and tortoises [14]. The first detection of a chelonian herpesvirus dates back to the mid 70's with the discovery of *Chelonid herpesvirus* 1 (ChHV1), known as "Gray patch disease-associated herpesvirus". The disease consisted in a multifocal to coalescent necrotizing and ulcerative dermatitis observed mainly in young green turtles (*Chelonia mydas*) [15]. *Chelonid herpesvirus* 2 and 3 (ChHV2, 3) were detected in fresh water turtles and more specifically in Pacific pond turtles (*Clemmys marmorata*-ChHV2) [16] and in painted turtles (Chrysemis picta-ChHV3) [17] with necrotizing hepatitis. *Chelonid herpesvirus* 4 (ChHV4) was detected in Argentine tortoises (*Geochelone chilensis*-ChHV4) with glossitis, rhinitis and pharyngitis [18]. Additional four herpesviruses were either detected or isolated from tortoises and were named *Testudinid herpesviruses* (TeHV1, 2, 3 and 4). TeHV1, 2 and 3 were associated with pathology mainly in tortoises of the genus *Testudo* (TeHV1 and 3) and in desert tortoises (*Gopherus agassizii*) (TeHV2) [14], whereas TeHV4 was detected in a clinically healthy bowsprit tortoise (*Chersina angulata*) [14, 19–21]. *Chelonid herpesvirus* 5 and 6 (ChHV5, 6) are two other sea turtle herpesviruses associated with a debilitating neoplastic disease called fibropapillomatosis (mainly in green and loggerhead-*Caretta caretta*-sea turtles) and with a multisystemic disease (in green turtles), respectively [22–25]. Two additional herpesviruses have been discovered in sea turtles and have been named loggerhead genital herpesvirus and the loggerhead cutaneous herpesvirus and they are responsible for multisystemic lesions in loggerhead sea turtles [13]. In the last few years an increasing number of

novel herpesviruses have been detected in fresh water turtles. These include a herpesvirus infecting the Australian Krefft's river turtles (*Emydura macquarii* kreftii) associated with proliferative and ulcerative tegument lesions [26] and Pelomedusid herpesvirus 1 in a clinically healthy West African mud turtle (*Pelusios castaneous*) [27]. *Emydid herpesvirus* 1 is a novel chelonian herpesvirus detected in an eastern river cooter (*Pseudemys concinna concinna*) with viral inclusions in the hepatocytes [28] and in both diseased (with pneumonia, hepatitis and splenitis) and clinically healthy northern map turtles (*Graptmeys geographica*) along with asymptomatic painted turtles [29]. Glyptemis herpesvirus 1 and *Glyptemys herpesvirus* 2 were recently detected in asymptomatic bog (*Glyptemis muhlenbergii*) and wood turtles (*Glyptemis insculpta*), respectively, whereas *Emydid herpesvirus* 2 was found in bog turtles and in a spotted turtle [30]. At least two herpesviruses have been detected in box turtles. Terrapene herpesvirus 1was detected in captive eastern box turtles (*Terrapene carolina carolina*) with stomatitis and glossitis [31], whereas Terrapene herpesvirus 2 was recently identified in an eastern box turtle coinfected with a spirorchid trematode and with papillomatous growths [32]. Despite the existence of all the chelonian herpesviruses described above, to date the only genome sequenced is that of ChHV5, a *Scutavirus* [22]. Only a few hundred nucleotides (nt) of DNA sequence belonging to the DNA polymerase (DNApol) gene have been determined for other herpesviruses infecting reptiles [33], accounting for less than 0.01% of their entire predicted genomes. These short sequences have been used to unravel the phylogenetic relatedness of reptilian herpesviruses. However, the relatively high conservation of the DNApol gene among herpesviruses, and more specifically of the selected PCR-amplified-region, has hampered any higher resolution regarding diversity of these viruses beyond the genotype. *Testudinid herpesvirus* es are important pathogens that have been detected in the chelonian family Testudinidae (tortoises) [14, 19–21]. Of the three genotypes, associated with obvious pathology, TeHV1 has been detected most frequently in Horsfield's tortoises (*Testudo* [*Agyonemis*] *horsfieldii*) having stomatitis and glossitis [14]. TeHV2 has been identified in North American desert tortoises [20, 21]. Severe stomatitis, glossitis and pneumonia have been observed in a TeHV2-infected captive desert tortoise along with pneumonia [21]. TeHV2 has been shown to serologically cross-react by ELISA with TeHV3 suggesting the existence of similar antigenic epitopes in the two distinct genotypes [20, 21]. Consistently, the partial sequence of the TeHV2 ribonucleotide reductase gene (large subunit), a conserved gene in herpesviruses, shared 79% identity with the homologous TeHV3 gene [21]. TeHV3 is considered the most pathogenic of the known TeHVs and it has been detected in several species of tortoises associated with stomatitis and glossitis although it appears to be overrepresented in

Greek tortoises (*Testudo graeca*) [14]. The pathology of TeHV3 has been thoroughly characterized in a transmission study in Greek tortoises infected with the type-strain 1976/98 with the virus inducing a disease whose severity was viral-load-dependent [34]. The classic clinical signs associated with the disease including stomatitis, mono- and bilateral recurrent conjunctivitis and oral discharge were reproduced in the experimentally infected tortoises [34]. In this study, the virus showed prominent neurotropism and indirect evidence of latency. Furthermore, the severity of the disease appears to vary with the species of tortoise infected. For instance, TeHV3 infection in the Greek tortoise is generally characterized by low mortality, whereas a severe, acute to sub-acute disease with high mortality has been observed in Hermann's tortoises (*Testudo hermanni*) [14].

Although numerous serological and molecular tests have been developed to diagnose exposure and infection with TeHV3 [33, 34–38], none of these tests can predict the virulence of the strain or whether one or several strains are involved within a single outbreak. To better understand the biology of this virus, we decided to sequence the complete genome of the TeHV3 type-strain 1976/96 [34, 36], the best characterized TeHV3 strain to date, to provide a complete reference genome sequence for TeHV3. Knowledge of this complete sequence in synthesis with further phylogenetic and experimental data would facilitate a better understanding of the host-pathogen relationship in chelonians and the development of more refined diagnostic tests contributing to predict the outcome of an outbreak. In addition, in this study we selected the sequence of the glycoprotein B (gB), gene as a tool to perform a higher resolution phylogenetic analysis on TeHV3 strains. The choice of the gB gene over other TeHV3 genes was based on its key features as an ideal phylogenetic marker including conservation among herpesviruses and sequence variability secondary to its antigenicity [39, 40]. These characteristics are expected to refine the resolution power of the viral DNApol sequence since the protein encoded by this gene is expected to be under a negative evolutionary pressure because of strong functional constraint. As we will show, the gB sequence allowed us to separate TeHV3 strains into at least two distinct genogroups. Moreover, we present preliminary data consistent with distinct genogroup-associated pathology in naturally and experimentally infected tortoises. We provide the first evidence of homologous recombination in a reptilian herpesvirus with indirect proof of a single tortoise infected with multiple strains of TeHV3. These new data will be fundamental for further investigations on host-pathogen interactions in chelonians and for tortoise conservation around the world.

In this manuscript we also propose to uniform the nomenclature of the TeHVs strains by presenting in the following order: the abbreviation of the country where the strain originated, followed by the original number assigned to the strain and the last two digits corresponding to the year of isolation. According to this procedure, the type strain 1976/96 will be renamed as (TeHV3)-US1976/98. All the other TeHVs strains investigated in this study will be named accordingly.

Material and Methods:

Genome sequencing

The TeHV3 strain US1976/98 was grown on Terrapene heart cells (TH-1;ATCC-CCL 50 Sub-line B1; American Type Culture Collection, Rockville, MD, USA), harvested and pelleted as previously described [36]. Total DNA was extracted using the DNAeasy kit (Qiagen Hombrechtikon, Switzerland). A total of 3.5µg of viral DNA was delivered to a biotechnology company (Fasteris SA, Geneva, Switzerland) for next generation sequencing (NGS). The viral DNA was processed using the Illumina technology for NGS. Briefly, the DNA was fragmented to produce short DNA inserts to build a DNA template library with each of the inserts carrying universal adaptors. Following quality control, the library was sequenced with the Illumina HiSeq 2000 to obtain 50 bases long single-end reads. De novo genome assembly was performed using the VELVET software (http://www.ebi.ac.uk/~zerbino/velvet/). To optimize the assembly, several values of k-mers were tested. To evaluate the quality of the assemblies, the following indicators were computed: sum of the contigs (DNA consensus sequences obtained by overlapping shorter DNA sequences) lengths, number of contigs, N50 (the length of the smallest contig in the set that contains the fewest (largest) contigs whose combined length represents at least 50% of the assembly) [41], number of contigs to reach the N50, average and maximum lengths of the contigs, and the number of reads from the library that can be mapped on the contigs. The assembly with the highest N50 and percentage of mapped reads was then chosen.

Bridging of the contigs was performed using viral sequences obtained from two previously generated sub-genomic DNA libraries (HindIII and EcoRI) [10] [34]. Following the assembly, multiple PCRs spanning 1–7.5Kb were performed to assess the junctions between contigs and the overall correctness of the assembly of the whole genome. Sequencing of the PCR products was carried out when the amplicon obtained was not consistent with the expected size derived from the sequenced genome. Finally, the single-end Illumina reads were mapped to the obtained genome sequence using BWA (Burrows-Wheeler aligner (http://bio-bwa.sourceforge.net)) [42]; the remapping was visualized with IGV (Integrative Genomic Viewer 2.3.34) [43]. For open reading frame (ORF) identification the program "ORF finder" (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used and each putative ORF was manually assessed using the program "BLAST" (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene identity was attributed according to the best hit provided by BLAST. An arbitrary cutoff corresponding to predicted genes encoding for at least 90 amino acids (aa) was selected when no homology could be found with any other known herpesviral genes using BLAST. Detection of the tandem repeats was carried out using the software "Tandem repeat finder" [44], using standard settings.

Genome comparison.

The TeHV3 genome was compared with that of *Chelonid herpesvirus* 5 (ChHV5), the only other available genome of a chelonian herpesvirus [22] using the program EasyFig v2.1 [45] with standard settings. Briefly the annotation files of the two genomes (HQ878327-ChHV5 and KT008627-TeHV3) were converted by the software into the corresponding tBLASTX files [45]. The graphic output of the comparison was then generated. Color tones from light gray to black were selected for the direct matches spanning from low to high, respectively, and orange to red for the inverted matches spanning from low to high, respectively.

Animals and pathology

A retrospective investigation was carried out on selected formalin-fixed, paraffin embedded (FFPE) tissues obtained from ten tortoises necropsied from 1999 to 2009 and stored in the archive of the Centre for Fish and Wildlife Health (FIWI) of the University of Bern, Switzerland.

All the tortoises selected for the study were captive animals and were either diagnosed as presumptively infected with TeHV or carrying compatible lesions based on having at least one of the following criteria: 1) eosinophilic to amphophilic intranuclear inclusions in at least one of the examined tissues (= presumptively infected); 2) presence of diphtheronecrotic stomatitis and/or glossitis (= compatible lesion); and 3) pneumonia (= compatible lesion). All these tortoises died of natural causes, with the exception of Z02/1970 that was humanely euthanized by the attending clinician because of very poor prognosis with an overdose of sodium pentobarbital performed by intra vascular injection. None of these tortoises was included in experimental studies. Two additional tortoises (PN186/12, PN13/08), selected using the above criteria, were provided by the University of Milan (Italy) along with a third tortoise (PN191/12) that despite absence of consistent lesions was also included in the study because it came from the same die off as PN186/12. A tortoise with a diphtheronecrotic stomatitis (S12/1458) submitted for necropsy to the Institute of Animal Pathology (ITPA) of the University of Bern was also included in the study. The three tortoises provided from the University of Milan and the tortoises submitted to the ITPA also died of natural causes and were not part of any transmission study. Finally, a tortoise (TG4/1998) experimentally infected with the TeHV3 type-strain US1976/98 with well-characterized associated pathology [34] was also considered for this study. This tortoise was part of a transmission study carried out previously. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida and the tortoise was humanely euthanized with an overdose of sodium pentobarbital performed by intracoelomatic injection [34]. All the tissues available from all the tortoises were reviewed by two of us (FCO and MT). The following data was obtained for each tortoise: 1) time of death; 2) sex; 3) species; 4) age; 5) geographical origin 6) presence of any inflammatory, necrotic or degenerative lesions in any of the sections observed using light microscopy, and presence and location of the intranuclear inclusions. No live animals were in-

cluded in this study. A detailed list of the tortoises included in this study including the identification numbers of each animal is provided in Table 1.

Tortoise ID	TeHV Strain ID	Species	Year	Month	From	TeHV Diagnosis	Weight	Sex	Main pathology findings
Z02/1970	CH1970/02	*T. hermanni*	2002	April	CH	Negative	1Kg	F	Glossitis
Z03/1690	CH1690/03	*T. hermanni*	2003	March	CH	Negative	2.1Kg	F	Stomatitis
Z07/1611	CH1611/07	*T. hermanni*	2007	April	CH	Positive	0.37Kg	F	Glossitis**,pneumonia, meningoencephalitis
Z06/2360	CH2360/06	*T. hermanni*	2006	June	CH	Positive	0.39Kg	M	Stomatitis and glossitis**
Z01/2053	CH2053/01	*Testudo. sp*	2001	April	CH	Positive	1.15Kg	N/A	Glossitis**, lymphoid depletion, pulmonary edema and splenitis
Z08/5132	CH5132/08	*T. hermanni*	2008	December	CH	Negative	0.02Kg	F	Pneumonia, enteritis, lymphoid depletion, nephritis, esophagitis and epicarditis
Z00/7204	CH7204/00	*S. pardalis*	2000	December	CH	Positive	2.7Kg	M	Glossitis**, hepatitis, enteritis**, pneumonia**, meningitis, tracheitis and vasculitis**
Z03/6883	CH6883/03	*T. hermanni*	2003	October	CH	Positive	0.9Kg	M	Glossitis**, stomatitis, hepatitis, pneumonia**, meningitis, perivasculitis
Z07/2313	CH2313/07	*T. hermanni*	2007	June	CH	Positive	0.13Kg	F	Stomatitis, glossitis**, lymphoid depletion
Z01/3429	CH3429/01	*T. hermanni*	2001	June	CH	Positive	0.09Kg	M	Glossitis**, hepatitis, pneumonia, Ttacheitis, rhinitis, lymphoid depletion and necrosis
S12/1458	CH1458/12	*T. hermanni*	2012	March	CH	Positive	0.346Kg	M	Stomatitis**, lymphoid depletion
TG4/1998*	US1976/98	*T. graeca*	1998	August	USA	Positive	0.8Kg	M	Stomatitis (experimental infection)
PN186/12	IT186/12	*T. hermanni*	2012	N/A	IT	Negative	0.07Kg	M	Tracheitis, nephritis, gastritis
PN191/12	IT191/12	*T. graeca*	2012	N/A	IT	Negative	0.115Kg	F	Ovarian neoplasia
PN13/08	IT13/08	*T. horsfieldii*	2008	Fall	IT	Positive	N/A	N/A	Pneumonia**, tracheitis, enteritis, stomatitis

N/A = Not available
* = This tortoise was euthanized during a previous study.
** = Lesions with intranuclear inclusions.
Year and months refer to the time of death of the tortoises.
TeHV Diagnosis = Positive or negative cases of herpesvirus infection based on the presence or absence, respectively, of the characteristic intranuclear inclusions in stained tissue sections. TG4/1998 is the only exception since although no inclusions were observed in the examined tissues, the tortoise was known to be infected with TeHV3 given that was part of a group of tortoises experimentally infected with US1976/98 [34].

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Table 1: Tortoise and TeHV strains.

Virus isolation

Virus isolation was attempted from fresh tissues obtained from tortoises PN186/12 and PN191/12 (from the University of Milan, Italy) and from an oral swab from tortoise S12/1458 (from the ITPA of the University of Bern, Switzerland). Tissues and swabs were processed for viral isolation by inoculation onto TH-1 cells at 28°C as previously described [34]. Cell cultures were monitored daily for the detection of cytopathic effects (CPE). The type-train 1976/96 was already available from previous studies [34, 36].

TeHV3 strain detection and partial characterization of the gB gene

Total DNA was extracted either from infected cell cultures (strains 1976/96, CH1458/12 and IT191/12) or from FFPE tissue blocks containing at least a section of the tongue and/or oral mucosa or lung from all the remaining tortoises in the study. Three 20 µm sections were obtained from each of the selected paraffin blocks and placed into an RNAse/DNAse-free 1.5 ml Eppendorf tube. Total DNA was extracted from each set of paraffin slices with the FFPE DNA extraction kit (Qiagen Hombrechtikon, Switzerland).
Total DNA was also extracted from infected cell cultures as previously described [34] and quantified with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The complete gB gene of strains US1976/98, CH1458/12 and IT191/12 was amplified by PCR with a forward (gBTeHV3FW) and a reverse (gBTeHV3RV) primers (Table 2) which were designed on the basis of the sequence available from the US1976/98 genome (KT008627). The PCR reaction-mix contained 5 µl of 2 µM forward and reverse primers, 1.25 µl of each 10 mM dNTPs (Promega, Madison, WI, USA), 5 µl of 10x PCR buffer, 1 µl of PFU II Ultra DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) 250 ng of total DNA template and double distilled water (Promega, Madison, WI, USA) up to 50 µl. PCR reactions were carried out in a DNA engine thermal cycler (MJ Research, Waltham, MA, USA) and comprised an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles comprising a denaturation step at 95°C for 30 seconds, an annealing step at 50°C for 30 seconds and an elongation step at 68°C for 1 minute followed by a final elongation step at 68°C for 10 minutes. Given that FFPE tissues undergo DNA fragmentation, no full amplification would have been possible with a single PCR reaction. Consequently, either full or partial gB gene amplification from the paraffin blocks-derived DNA was carried out with a series of sequential and overlapping PCR reactions, which were set up with each of the subgroup of primer sets listed in Tables 2 and 3, respectively. More specifically, the pairs FWA and RVA, FW1 and RVB, FW2 and RVC, FW3 and RVD, FW4 and RVE, FW5 and RVF, FW6 and gBTeHV3RV were used to amplify several DNA fragments covering the full length gB of strain CH6883/03, while the primer pairs FW4 and RVE, FW5 and RVF, FW6 and gBTeHV3RV were used to amplify the 3' half portion of the gB gene of all the other paraffin-blocks-derived strains in the study. Each of the PCR reaction mixes contained 0.25 µl of 10 µM forward and reverse primers, 12.5 µl of hot-start master mix (Qiagen AG, Hombrechtikon, Switzerland), 250 ng of total DNA template and double distilled water (Promega, Madison, WI, USA) up to 25 µl. The reaction mixes were placed in a DNA engine thermal cycler (MJ Research, Waltham, MA, USA) and after an initial denaturation at 95°C for 15 minutes (hot start), 45 cycles comprising denaturation at 95°C for 1 minute, annealing at 50°C (FWA and RVA, FW4 and RVE, FW5 and RVF) or 52°C (FW1 and RVB, FW2 and RVC, FW3 and RVD, FW6 and gBTeHV3RV) for 30 seconds, an elongation at 72°C for 30 seconds, followed. A final step comprising 10 minutes extension at 72°C was added. A summary of the primer pairs used for the gB gene amplification and cycling conditions is listed in Tables 2 and 3.

Primer name	Primer sequence (5'-3')
FWA	TTTGGTTATCATATTAGGAGCG
RVA	ACGTCTATTATTTCGTCACGC
FW1	GAGCATAGGTCATAGAACAACTATACG
RVB	GTCCTCGTTCATAGTTTCGG
FW2	AGATGTCTCCATTCTACGATAGAACC
RVC	CAGATGAGTATCCTTGTACCG
FW3	CCGACCGAAGGCAAAAAAGAAATAGA
RVD	AAACGCGACGCTTCATTATGG
FW4	ACAATTAAATAAAATAAATCCC
RVE	ATATACGTGCTTACTTCTGGG
FW5	ATAATTTTGTTAGGATGGTTC
RVF	TAAAATGATAGTAAATCCTCC
FW6	TTTTTCAGTAACCCGTTCGGAGG
gBTeHV3FW	AAAATGATCATGTGGTTATCGTT
gBTeHV3RV	AAAATTATTGGGAGGAATCGTCTATCT

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Table 2: PCR and sequencing primers

Glycoprotein B amplification conditions			
gB amplification	**Primer sets**	**Amplicon size (nt)**	**Cycling conditions**
Full (from fresh tissues)	gBTeHV3FW-gBTeHV3RV	2,484*	95°C for 1' + 40 cycles = 95°C for 30", annealing at 50°C for 30" + 68°C for 1'. Final elongation step at 68°C for 10'
Full (from formalin-fixed tissues)	FWA-RVA	524*	95°C for 15' (hot start)+ 45
	FW1-RVB	409*	cycles = 95°C-1' + annealing
	FW2-RVC	413*	at 50°C (FWA and RVA,
	FW3-RVD	409*	FW4 and RVE, FW5 and
	FW4-RVE	405*	RVF) or 52°C (FW1 and
	FW5-RVF	389*	RVB, FW2 and RVC, FW3
	FW6-gBTeHV3RV	387*	and RVD, FW6 and
Partial	FW4-RVE	405*	gBTeHV3RV) for 30", +
	FW5-RVF	389*	72°C for 30". A final
	FW6-gBTeHV3RV	387*	elongation step for 10' at 72°C

* Expected amplicon size including primers

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Table 3: Glycoprotein B gene PCR amplification conditions

Phylogenetic analyses

Multiple phylogenetic analyses were carried out. The first analysis (***1***) was based on the partial aa sequence of the DNApol and gB proteins, respectively, encoded in the genome of the TeHVs strains included in this study (N = 15).

These sequences were compared to homologous sequences available from other herpesviruses (TeHV1-AB047545.1, TeHV2-AY916792.1, TeHV4-GQ222415.1, ChHV5-AF239684.2, ChHV6-EU006876.1 and GaHV1- AF168792.1 for the DNApol; and ChHV5-AAU93326, ChHV6-AAM95776 and GaHV1-YP_182356 for the gB sequences). The second analysis (***2***) was based on the partial nt sequences of the gB gene of all the TeHV3 strains included in this study (n = 15), which were compared to the homologous gene of other herpesviruses (ChHV5-AAU93326, ChHV6-AAM95776 and GaHV1- YP_182356).
The third analysis (***3***) was carried out using the complete aa sequence of the gB protein of the reference strain (US1976/98), which was compared to homologous sequences of 36 other herpesviruses. Sequence alignment was carried out using multiple software packages (ClustalW, ClustalW2, TCoffee, Mafft L and Muscle) [*46–49*] for phylogenetic analysis ***1***, whereas ClustalW was used for analysis 2 and 3. Maximum likelihood phylogenetic trees were obtained using MEGA6.0–6 [*50*] software package with 500 bootstrap replications, Jones Taylor Thorton replication method, uniform rates among sites and very strong branch swap filter.

Recombination analysis

Assessment of recombination events of the gB gene was carried out with the Recombination Detection Program (RDP4) (Version 4.46) [51] using standard settings and feeding the software with two of the predicted parent strains (US1976/98 and CH6883/03) together with the predicted recombinant strain (CH3429/01). The methods used for the recombination assessment were those included in the software package and included RDP, GENECONV, BootScan, MaxChi, Chimera, SiScan, PhylPro, LARD and 3Seq, all operating under standard settings.

Results:

Sequence analysis of the TeHV3 genome

Next generation sequencing (NGS): A total yield of 1.598 mega bases (Mb) was obtained with 31,959,585 clusters. 95.8% of the bases had a Q-score (measure of base-calling accuracy) larger than 30. The *de novo* assembly of the reads led to a total of 24 contigs spanning from 107 to 64,501 nt long and accounting for 140,195 nt (N50 of 23,603 nt). The average base coverage (depth) was 8,984. Bridging of the contigs was carried out by PCR and with the aid of the sequencing information derived from the *Hind*III and *Eco*RI subgenomic libraries as previously mentioned. The final sequence of the genome comprised 150,080 nt (Fig 1).

The TeHV3 genome is composed of a unique long (UL) and a unique short (US) region, with the US flanked by inverted repeats consistent with a type D arrangement similar to that of *Human herpesvirus 3* (HHV3-VZV) and the recently described genome of ChHV5 [22]. The GC content was 45.8%.

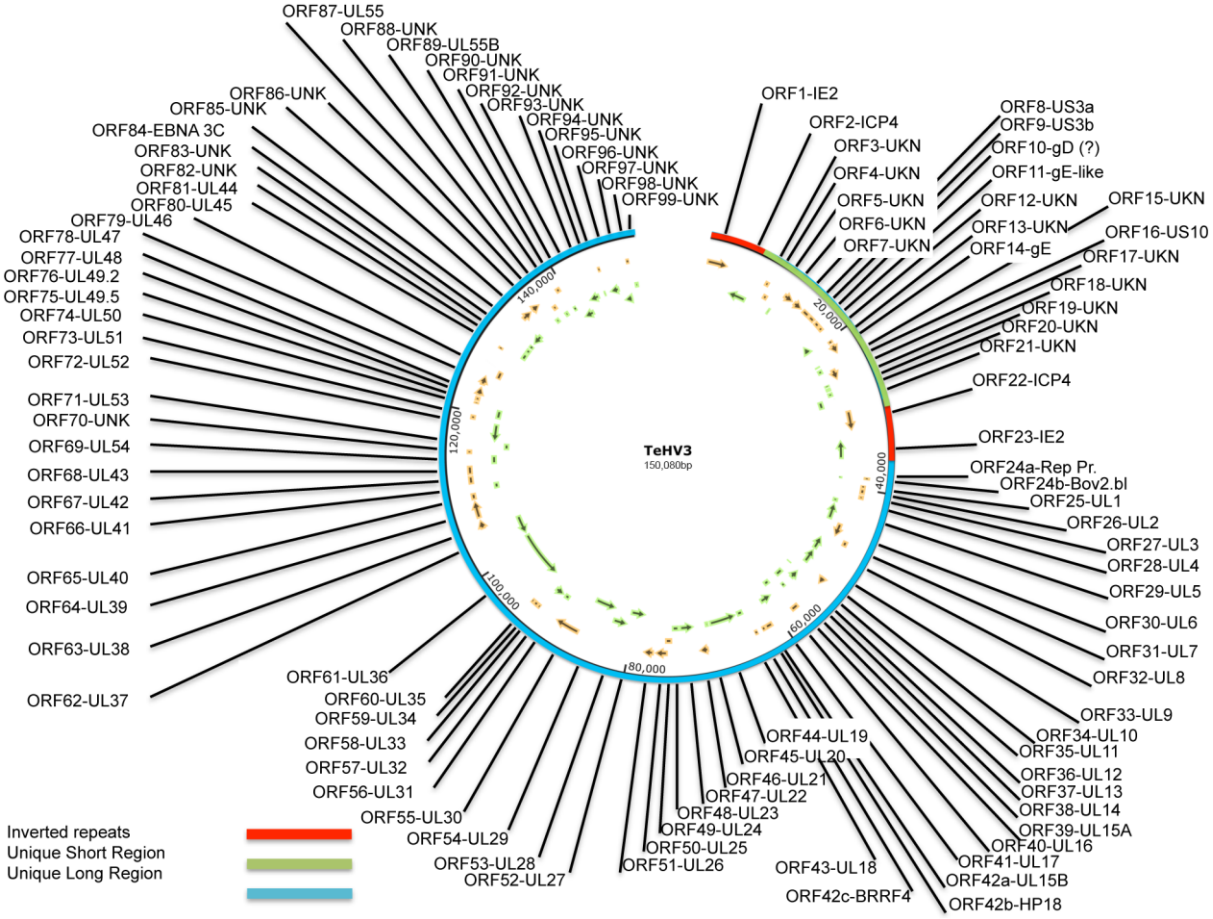

Figure 1: TeHV3 genome map. The TeHv3 genome is composed of a unique longue region (UL) (blue bar) and a unique short region (green bar) flanked by two inverted repeats (red bars). The progressive numbers and homologous herpesvirus genes of the identified sense (orange) and antisense (green) open reading frames (ORFs) are shown in the map (SnapGene Viewer-V2.7; www.snapgene.com).

Analysis of the sequence of the unique long (UL) region.

The length of the available UL region was determined to be 112,838 nt (position 37,243 to 150.080 of the genome). The putative 5' and 3' ends of the UL region were identified as those regions immediately contiguous to the inverted and terminal repeats, respectively. The putative 5' end of the UL region was obtained by NGS complemented with the sub-genomic library approach, whereas the sequence of the putative 3' end was obtained by sequencing a PCR amplicon bridging the 5' end region of the terminal repeat and the 3' end of the UL region. This approach was attempted given that herpesvirus genomes are known to adopt a closed circle configuration within a few hours after the start of replication [52]. By harvesting the total DNA of early-infected TH-1 cells, we could successfully amplify the bridging regions between the terminal repeat and the 3' end of the UL region. The actual putative 3' end of the UL region was considered to be the last nucleotide prior to the beginning of the sequence corresponding to the reverse and complement of the 3' end of the internal inverted repeat. Multiple PCRs confirmed the assembly obtained.

The predicted UL region comprises at least 79 ORFs; 37FW, 42RV, with 35 partially overlapping. Sixty-two of them showed variable degrees of similarity with known herpesvirus genes, ranging from 38 to 79% for the UL47 (*Psittacid herpesvirus 1*) and UL 45 (*Ateline herpesvirus 3*) homologues, respectively. The remaining ORFs could not be associated unambiguously to a known herpesvirus gene (Table 4 and Figs 1 and 2).

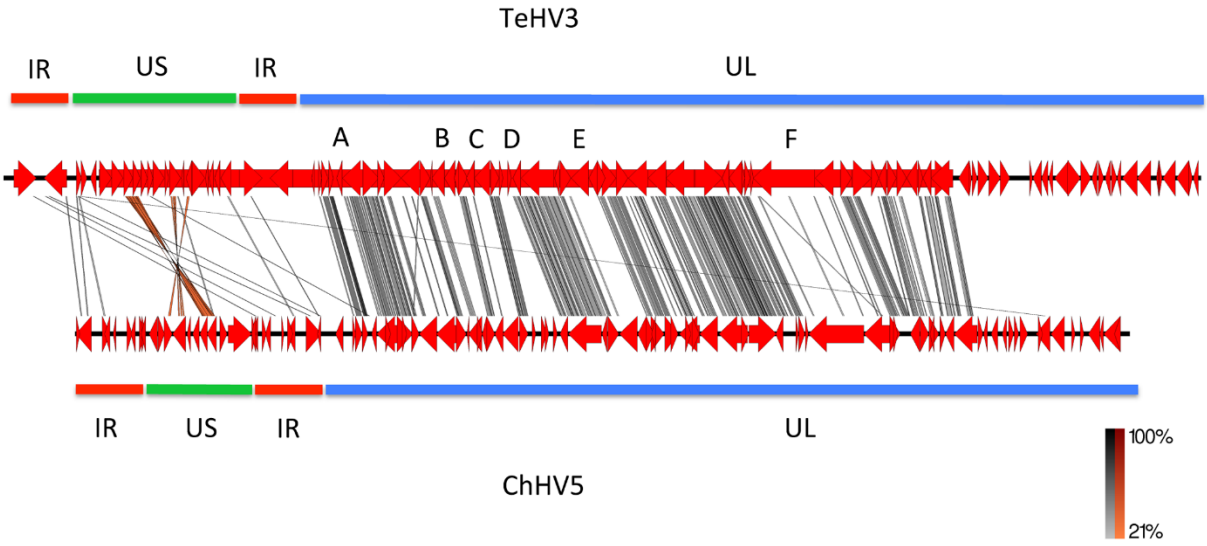

Figure 2: TeHV3 and ChHV5 genome comparison. The graphical comparison between TeHV3 and ChHV5 genomes obtained with the EasyFig1 software is shown in the figure. The genomes of TeHV3 and ChHV5 are depicted in red, with a black backbone. Similar regions are connected with grey (low) to black (high) (direct sequences) and orange (low) to red (high) (inverted sequences) bars. Green, blue and red bars highlighting the US and UL regions and of the inverted repeats (terminal and internal) of each genome, respectively are also shown. Letters A, B, C, D, E and F correspond to regions of the genomes that show no similarities within the conserved UL block.

ORF	HV Homologue	Protein	Function/ description	Similarity*	Position	Length (nt)	Frame
1	HHV6	IE2	Transactivator	40%77/188	1,097–3,916	2,820	+
2	ChHV5	ICP4	Transactivator	58%41/70	4,897–7,737	2,841	-
3	N/A	UNKN	N/A	N/A	8,948–9,580	633	+
4	N/A	UNKN	N/A	N/A	9,555–10,280	726	+
5	N/A	UNKN	N/A	N/A	10,704–11,444	741	-
6	N/A	UNKN	N/A	N/A	11,864–13,348	1,485	+
7	N/A	UNKN	N/A	N/A	13,041–14,798	1,398	+
8	ChHV5	US3a	Serine/Threonine Kinase	54% 181/333	14,902–15,993	1,092	+
9	ChHV5	US3b	Putative cyclin-dependent kinase 2	50% 153/305	16,026–16,964	939	+
10	ChHV5	gD	Cell receptor ligand	44%78/175	17,055–17,858	804	+
11	HPV2	gE-like	Unknown	41%58/141	17,874–18,542	669	+
12	N/A	UNKN	N/A	N/A	18,557–19,957	1,401	-
13	N/A	UNKN	N/A	N/A	20,069–20,512	444	+
14	ChHV5	gE	Host interaction and immunoevasion	41%159/387	20,634–22,031	1,398	+
15	N/A	UNKN	N/A	N/A	22,141–22,416	276	-
16	ChHV5	US10	Zinc-ion binding/ Tegument/capsid protein	54%106/194	22,683–23,429	747	-
17	N/A	UNKN	N/A	N/A	23,448–25,085	1,638	+
18	N/A	UNKN	N/A	N/A	25,127–25,489	363	-
19	N/A	UNKN	N/A	N/A	25,572–26,120	549	-
20	N/A	UNKN	N/A	N/A	26,268–26,969	702	-
21	N/A	UNKN	N/A	N/A	27,050–28,438	1,388	-
22	ChHV5	ICP4	Transactivator	58%41/70	29,506–32,346	2,841	+
23	HHV6	IE2	Transactivator	40% 77/188	33,327–36,146	2,820	-
24a	RFHVMn	Rep Protein	Unknown	38%34/88	38,525–38860	336	+
24b	BoHV6	Bov2.bl	Unknown	51%44/85	38,574–38,906	333	-
25	PsHV1	gL	Viral entry	58%33/56	39,402–39,856	384	+
26	ChHV5	UL2	UDG glycosilase	61%156/252	39,792–40,604	813	+
27	ChHV5	UL3	Phosphoprotein	72%154/211	40,685–41,266	582	+
28	ChHV5	UL4	Nuclear protein	50%86/172	41,682–42,365	684	-
29	ChHV5	UL5	DNA helicase/primase cpx	69%598/858	42,416–44,917,	2,556	-
30	ChHV5	UL6	Capsid protein	60%398/654	44,955–46,964	2,010	+
31	ChHV5	UL7	Virion egress protein	53%132/248	46,807–47,622	816	+
32	ChHV5	UL8	DNA helicase/primase cpx	44%336/749	47,627–49,777	2,151	-
33	AnHV1	UL9	Replication origin-binding protein	57%463/810	49,779–52,133	2,355	-
34	ChHV5	gM	Virion assembly and egress	66%279/418	52,145–53,383	1,239	+
35	HSV1	UL11	Tegument protein	62%27/43	53,433–53,672	240	-
36	ChHV5	UL12	Alkaline exonuclease	55%271/484	53,606–55,207	1,602	-
37	HSV1	UL13	Serine/threonine kinase	42%184/428	55,208–56,521	1,314	-
38	ChHV5	UL14	Tegument protein	58%58/100	56,443–56,976	534	-
39	ChHV5	UL15A	Putative DNA packing protein	64%214/330	56,975–58,090	1,116	+
40	ChHV5	UL16	Capsid binding protein	53%179/332	57,938–58,945	1,008	-
41	ChHV5	UL17	Virion Packaging protein	46%315/676	58,924–60,882	1,959	-

Table 4: TeVH3 Open reading fragments features.

ORF	HV Homologue	Protein	Function/ description	Similarity*	Position	Length (nt)	Frame
42a	ChHV5	UL15B	Terminase large subunit	74%267/359	60,986–62,050	1,065	+
42b	ChHV5	HP18	Hypothetical protein	43%34/79	62,069–62,523	453	+
42c	HHV4	BRRF4	BRRF4 protein	40%41/101	63,118–63,579	462	+
43	ChHV5	UL18/VP23	Capsid protein	65%211/321	63,784–64,746	963	-
44	ChHV5	UL19	Major capsid protein	72%993/1,373	64,821–68,930	4,110	-
45	ChHV5	UL20	Multifunctional essential for fusion	75%139/184	69,023–69,613	591	-
46	ChHV5	UL21	Tegument protein	45%220/480	69,716–71,068	1,353	+
47	ChHV6	UL22/gH	Fusion/cell entry	74%332/445	71,088–73,289	2,202	-
48	ChHV5	UL23/TK	De novo DNA synthesis	48%168/345	73,322–74,389	1,068	-
49	BoHV1	UL24	Nuclear protein	52%94/178	74,371–75,345	975	+
50	ChHV5	UL25	Virion packaging	55%323/578	74,960–756,687	1,728	+
51	ChHV6	UL26	Protease	60%352/579	76,726–78,198	1,473	+
52	ChHV6	UL27/gB	Cell entry/Fusion	73%637/864	78,251–80,734	2,484	-
53	ChHV5	UL28/ICP18.5	DNA processing and packaging	64%475/742	80,731–83,022	2,292	-
54	ChHV5	UL29/ICP8	Major DNA binding protein	63%735/1,156	82,976–86,527	3,552	-
55	ChHV5	UL30	DNA polymerase	70%809/1150	86,651–90,049	3,399	+
56	ChHV5	UL31	Nuclear matrix protein	67%185/275	89,991–90,878	888	-
57	ChHV5	UL32	DNA cleavage/packaging	63%296/466	90,884–92,497	1,614	-
58	ChHV5	UL33	DNA processing and packaging	66%72/108	92,478–92,825	348	+
59	ChHV5	UL34	Inner nuclear membrane protein	53%143/269	92,827–93,555	729	+
60	PsHV1	UL35	Small capsid protein	52%24/46	93,565–93,909	345	+
61	ChHV5	UL36	Large tegument protein	44%942/2101	93,906–101,630	7,725	-
62	ChHV5	UL37	Capsid assembly	43%461/1051	101,696–104,866	3,171	-
63	ChHV5	UL38	Capsid protein	56%256/455	104,868–106,211	1,344	+
64	ChHV5	UL39	Ribonucleotide reductase L.s.	62%490/787	106,315–108,705	2,391	+
65	FeHV1	UL40	Ribonucleotide reductase S. s.	73%220/301	108,728–109,642	915	+
66	ChHV5	UL41	Virion host shutoff protein	62%212/338	109,637–110,596	960	-
67	ChHV5	UL42	DNA polymerase processivity factor	48%147/304	110,732–111,838	1,107	+
68	ChHV5	UL43	Membrane protein	45%178/393	111,932–113,122	1,191	+
69	EqHV3	UL54/ICP27	Transactivator	50%109/214	113,152–114,438	1,287	-
70	N/A	UNKN	N/A	N/A	114,557–115,162	606	+
71	ChHV5	UL53/gK	Intracellular fusion	64%227/353	115,194–116,234	1,041	-
72	ChHV5	UL52	DNA primase	55%490/880	116,231–118,951	2,721	-
73	PsHV1	UL51	Tegument protein	47%82/174	118,950–119,600	651	+
74	MeHV1	UL50	Deoxyuridine triphosphatase	50%82/163	119,642–120,985	1,344	-
75	BoHV6	ORF53/UL49.5	Tegument/envelope protein	48%48/100	120,984–121,286	303	+
76	AnHV1	UL49.2	Tegument protein	60%59/98	121,352–121,990	639	+
77	SuHV1	UL48	Tegument protein	43%163/378	122,083–123,369	1,287	+
78	PsHV1	UL47	Tegument protein	38%95/247	123,467–124,888	1,422	+
79	AnHV1	UL46	Tegument protein	42%95/223	124,881–126,092	1,212	+
80	HHV3	UL45	Thymidylate synthetase	79%98/124	128,556–128,930	375	+
81	CeHV9	UL44/gC	Ligand/immunoevasion	45%104/228	128,974–130,026	1,053	-
82	N/A	UNKN	N/A	N/A	130,062–130,850	789	-
83	N/A	UNKN	N/A	N/A	130,954–131,490	537	-
84	HHV4	EBNA-3C	Nuclear protein	52%39/75	131,690–133,450	1,761	-
85	N/A	UNKN	N/A	N/A	133,338–134,915	1,578	+
86	N/A	UNKN	N/A	N/A	134,954–136,252	1,299	+
87	BoHV2	UL55a	Unknown	39%43/110	136,355–137,020	666	-
88	N/A	UNKN	N/A	N/A	137,152–137,841	690	+
89	ChHV5	UL55b	Unknown	44%28/63	138,014–138,670	657	-
90	N/A	UNKN	N/A	N/A	138,799–139,494	696	+
91	N/A	UNKN	N/A	N/A	139,517–140,005	489	-
92	N/A	UNKN	N/A	N/A	140,190–142,040	1,851	-
93	N/A	UNKN	N/A	N/A	142,037–143,752	1,716	-
94	N/A	UNKN	N/A	N/A	144,464–144,760	297	-
95	N/A	UNKN	N/A	N/A	144,780–145,166	387	+
96	N/A	UNKN	N/A	N/A	145,269–146,831	1,563	-
97	N/A	UNKN	N/A	N/A	146,976,-148,493	1,518	-
98	N/A	UNKN	N/A	N/A	148,510–148,968	459	+
99	N/A	UNKN	N/A	N/A	149,077149,700	624	-

*The number at the denominator indicates the total length of the portion of the herpesviral homologous protein being compared by BLAST with TeHV3. The number at the numerator indicates the actual number of the amino acid residues of TeHV3 showing similarities with the compared portion of the homologous protein. The percentage summarizes the overall similarity between TeHV3 and the specific herpesvirus homologous protein for the specific motif considered.

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Table 4 (continue): TeVH3 Open reading fragments features

The UL ORFs are largely co-linear to the homologous genes of *Human herpesvirus 1* (HSV1), in particular from UL1 to UL43, while the segment spanning from UL53 to UL44 has an inverted orientation compared to HSV1. Two genes share homology with HSV1 UL55 (UL55a and UL55b), with 74% nt identities between each other, suggesting gene duplication. TeHV3 encodes for the thymidylate synthetase (UL45), whose homologue is found only in *Varicella zoster virus* (VZV) of all the *Alphaherpesvirinae*, whereas is common in *Gammaherpesvirinae*. The longest gene identified in the UL region was the homologous of HSV1 UL36, encoding for the large tegument protein. This gene is 7,725 nt long and encodes for a protein 2,574 aa long. In contrast, the shortest gene identified within the UL region is the homologous gene of HSV1 UL11. The gene is 240 nt long and encodes for a 79aa long protein. Finally, upstream of the homologous gene of HSV1 UL1 is a 333nt ORF (ORF24b) encoding for a predicted 110 aa long protein that shares 51% similarity with a motif of a predicted protein with unknown functions encoded by *Bovine herpesvirus 6* (Table 4). Interestingly, almost entirely overlapping with ORF24b is ORF24a, which is another predicted ORF whose encoded predicted protein shares very limited similarities with a protein encoded by the retroperitoneal fibromatosis-associated herpesvirus (Genbank AGY30683) (Table 4).

Interestingly, the UL region of ChHV5, the closest related herpesviral genome to TeHV3, lacked several genes compared to TeHV3. More specifically, the HSV1's UL13, UL40, UL44 through 51 and UL54 through UL56 homologous genes could not be detected in the ChHV5 genome [22]. In contrast, homologues of all these genes except for UL56 were identified in the TeHV3 genome. UL13 encodes for a serine-threonine protein kinase, whereas UL40 encodes for the small subunit of the ribonucleotide reductase. UL44 encodes for the glycoprotein C, a surface membrane protein which mediates immune evasion *in vivo* [53], while UL45 encoded for thymidylate synthetase. UL46, UL47, UL48 UL49.2, UL49.5 and UL51 encode for tegument proteins. UL50 encodes for a metabolic enzyme (dUTP diphosphatase).

UL54 encodes for a transcriptional regulator (similar to ICP27), while UL55 encodes a protein with unknown functions. Ackermann and colleagues [22] reported that all the homologues genes from HSV-1 that are missing in ChHV5 in comparison with TeHV3 are known to be non-essential for herpesvirus replication *in vitro*. The biological meaning of these different sets of genes in TeHV3 is currently unknown. Table 4 provides a summary of all the comparison data.

Analysis of the sequence of the unique short (US) region.

The US region was identified as the portion of genome between the terminal and inverted repeats. It is predicted to be 20,374 nt long (position 8,435 to 28,808 of the genome) and comprises at least 19 ORFs (11 FW, eight RV, two overlapping). Only five of the detected ORFs shared a relatively significant similarity with other known herpesviral genes (ORF8 and 9 with US3; ORF10 with gD; ORF14 with gE; ORF16 with US10) (Table 4). There are two genes that share high similarity with HSV1 US3, US3a (ORF8) and US3b (ORF9) (see below). ORF8 and 9 do not share any significant similarity between each other (only 10 positive aa between the two sequences), suggesting that an origin by gene duplication is unlikely. Fifty-four per cent sequence similarity was observed between the TeHV3 ORF8 encoded protein and ChHV5 US3 protein homologue, whereas 50% sequence similarity was observed between the TeHV3 ORF9 encoded protein and the putative cyclin-dependent kinase 2 of ChHV5. The predicted protein encoded by the ORF 10 of TeHV3 shared 48% sequence similarity with a 175 aa long motif of ChHV5 glycoprotein D (gD), a molecule that is known to serve as the major receptor-binding protein in a number of *Alphaherpesvirinae* [22] (Table 4).

The longest predicted gene detected in the US region encoded for a protein of 545 aa, identified as TeHV3 ORF17, that did not share any similarity with any gene of known herpesviruses. The shortest ORF detected in the US region was instead identified as TeHV3 ORF15 and encoded for a 91 aa long protein and did not show any similarity with other known herpesvirus genes. The highest similarity between the identified proteins encoded by the TeHV3 US region and those of other herpesviruses was observed for the homologue of HSV1 US3 (see above) and of US10, both with 54% similarity with the correspondent protein encoded by ChHV5 (Table 4). Otherwise, the lowest similarity was observed for the glycoprotein E (gE) (ORF14) and of a gE-like protein (ORF11), with 41% similarity for the homologous proteins encoded by ChHV5 and *Cercopithecine herpesvirus 6*, respectively. No similarities could be found between the other TeHV3 US encoded predicted proteins and those of other known herpesviruses. Overall, findings were similar to those described for the US region of ChHV5, except for the number of ORFs identified, which in ChHV5 were 11 versus the 19 identified in TeHV3. Table 4 provides a summary of all the comparison data.

Analysis of the sequence of the inverted repeats (Internal and terminal repeats).

The regions identified as putative inverted repeats are both 8,434 long (position 1 to 8,434 and position 28,809 to 37,242 of the genome). The beginnings and the ends of the inverted repeats were identified as those regions immediately contiguous to unique sequences of either the UL or US regions. The inverted repeats encode for at least two predicted proteins with motifs sharing 58 and 40% similarity with two known transactivators, ICP4 (ORF2 and 22) and IE-2 (ORF1 and 23),(Table 4).

No detectable latency associated transcript (LAT) encoding gene was detected, in contrast with ChHV5. Strikingly, a major difference in the number of the predicted ORFs was observed between the inverted repeat regions of TeHV3 and that of ChHV5, where 12 ORFs were identified. However, while in ChHV5, the cutoff selected for the detection of the ORFs was 40 aa, we considered only the ORFs predicted to encode at least 90 aa as mentioned above (Table 4). Finally, five additional putative ORFs encoding more than 90 aa were actually identified in the TeHV3 inverted repeats, however, they were almost entirely clustered within the ICP4 homologous coding sequence and all of them showed very low similarities only with very short motifs of known herpesvirus genes and consequently were not considered in the final list of TeHV3 putative genes.

Tandem repeats.

A total of 17 tandem-repeat regions were identified and the length of the repeat motifs ranged from 13 to 73 nt (Table 5). Four of them mapped within the UL region, eight in the inverted repeats, three in the US region, one bridging between UL region and the inverted repeat and one bridging between US and inverted repeat. In four of the repeated motifs up to 4 mismatches were observed. Two indels were also observed (Table 5).

Tandem repeat sequence	Motif Length (nt)	Position	Location
CAGACTCCGTCCGTTAGATTTTGTCAAATTCTGGTCGAGTCGAAC	41	241–359	Inv. repeat
ACGTAACCCTAGCTGCTCTAAGGGACA	27	627–720	Inv. repeat
TGTCGGCCACCCTGACTCTACCCCGCTCCGGCCTGCCCCC	40	4015–4839	Inv. repeat
ATGCATATCATTAAATATGGAGGAGGTTATGGAATGAGGGAAGTCGGCTCCGATTGGTAGTTGGG	73	7949–8347	Inv. repeat
GGTGGATATGGGTGGATGTATGTTTATGCATATCATTAAAAAT*[1]	43	8385–8563	Bridging 5'end US and inv. repeat
AAAAGCAAAACACTTGTATAAATTATCCAAT*[2]	31	16943–17010	US3 region
GGTTGGGGAGAACGGTCATAATTAGAGGGACCG	33	27528–27703	ORF 21
TATCCGAGTTACTACCCCCCTGTACCTGCTGGAAAT*[3]	36	27994–28312	3'end US
TTCCATAACCTCCTCCATATTTAATGATATGCATCTACCTGCCCCAACTACCAATCGGAGCCGACTTCCCTCA	73	28896–29294	Inv. repeats
CGGGGTAGAGTCAGGGTGGCCGACAGGGGGCAGGCCGGAG	40	32404–33228	Inv. repeats
GCTAGGGTTACGTTGTCCCTTAGAGCA	27	36523–36616	Inv. repeats
ATTTGACAAAATCTAACGGACGGAGTCTGGTTCGACTCGACCAGA	45	36884–37002	Inv. repeats
CCCTGGATCGGGCCC	15	38589–38844	ORF 24
ATATATAAATATA	13	62779–62971	UL15b/UL18 region
GACAACTTGAGGAGGAGGTGGTGGGTG	27	100095–100169	UL36
GGATCCTTAAACTAGATCCCTTTACATGTAGTATGTTACTATAAAAC	47	127865–128049	UL45/46 region
GGTGGGGTGATCGGAGGGGTAACCTCTTCT*[4]	30	131704–131989	UL44 region

*Presence of mismatches or indels or both in the repeated motifs

[1]One mismatch

[2]Four mismatches and one indel

[3]One mismatch

[4]One indel

Inv. repeats = Inverted repeats

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Table 5: Tandem repeats features.

Ambiguous sequencing results.

In the region comprised between the homologous genes to UL44 and UL55 we identified a tandem repeat region. The PCR bridging this repeated sequence yielded a product that appeared to be approximately 50 nt longer than the original predicted sequence. Several attempts to sequence that region failed. Similarly, the region across UL15b and UL18, which also contains repeated sequences, might be approximately 200 nt shorter than predicted by the original sequence.

The putative 3' end of the UL region comprises a 5.4 Kb contig that was originally assembled in the opposite orientation of the one then determined to be the correct one. The ambiguity was determined by the conflicting results of a short bridging PCR over the sequence gap, which could not be confirmed with a long bridging PCR. The orientation of the contig presented in the final sequence here was selected on the basis of PCR and sequencing results. Similarly, between the IE-2 and ICP4 homologues we identified a GC rich region 1,015 nt long with repeated motifs (tandem repeats). Interestingly, the PCR product spanning between the two genes over the GC rich region showed multiple bands ranging from approximately 900 to 700 nt, but all smaller than the expected product. However, when running a PCR positioning the primers distally from the GC rich region, an amplicon of the expected size was obtained. The pattern of the short bridging PCR results was very similar to that described by Hommelsheim and colleagues [54] when sequencing repetitive DNA sequences. In the context of a different research project, while screening subgenomic clones of TeHV3 strain US1976/98 obtained by partial digestion of the genomic DNA with *Sau3*AI we detected a repeated motif comprising alternating long series of single A, T, C or G (up to 22 nt long) that we could not find in the assembled genome. This repeated motif was contiguous with a sequence that was only partially matching that bordering a similar, but different repeated motifs at the beginning of the putative UL region. Blasting the novel identified nt motif (with the option "somewhat similar sequences" of BLAST) no match with any of the known herpesviruses was observed. Contrastingly, when the search was not restricted to herpesviruses only, identities up to 84% of portions of the sequence (up to 33%) with eukaryotic organisms (*Oryzias latipes*-HG313981.1) were observed. Finally, the original sequence of the TeHV3 genome was determined to be 170 nt longer than the one presented in this article. This additional sequence was located at the 5' end of the terminal repeat and it was one of the two motifs of a tandem repeat originally identified in that region. When assessing the assembled genome by the software IGV, a total of 12 ambiguous nt were observed across the entire genome. Of these, six clustered in the first 222 nt of the genome and two were in the last 20 nt of the genome. In particular in association with four of the nt ambiguities at the 5' end of the genome (in correspondence of the 5' end of the terminal repeat), we also observed an abrupt drop of the coverage. Interestingly this drop in coverage corresponds to the joining of the two tandem repeats described above (region between original nt 166 to 172). Multiple attempts of sequencing these regions were carried out, but the presence of repeated motifs did not allow us to conclusively resolve these ambiguities. Given the clustering of several sequence and as-

sembly ambiguities in the region across these two tandem repeats and that once the two tandem repeats were collapsed into just one, the assembly of that region appeared to be more robust, we considered that this 170 nt fragment might have represented a sequencing artifact and then it was removed. Following the editing of the sequence according to what described above, the inverted and tandem repeats, which originally differed in size for exactly 170 nt in length, were then both measuring exactly 8,434 nt, further supporting the editing described above. A possible insertion of a nt was instead detected at position 127,377, whereas a possible deletion of a nt was observed at position 131,553 of the genome. A drop of coverage of 30% between contiguous nt was seen at positions 37,191 and 37,242 but the assembly in that region appears solid and no further assessment was considered necessary.

Genome Comparison.

The graphic outcome of the software EasyFig1 (Fig 2), highlighted a prominent conservation of the arrangement of the genes clustering into the central portion of the UL region of the genome, although showing overall low similarity as suggested by the predominant light gray of the connecting lines. However, within this highly conserved portion of the genome, few regions showed absence of virtually any similarity. More specifically, the regions corresponding to the homologue of UL4 (A), UL12-13 (B), UL16 (C), in that between the UL15b and UL18 homologues (D) and in that corresponding to the UL22 (E) and UL36 (F) homologues, none or very limited numbers of connecting lines were observed. The extremities of the genomes showed a relative low or absence of similarities, with the most relevant clustering within the US regions of the genomes where low similarity-labeling color (orange) highlighted corresponding inverted matches (ORF 8-US3a, 9-US3b, 14-gE and 16-US10). No matches could be observed in the terminal portion of the putative UL regions of the two genomes.

Animals

A total of 15 tortoises were included in this study. Ten of them were diagnosed as presumptively infected by herpesvirus and five as carrying consistent lesions (or history = IT191/12) based on the criteria described above. The only exception was tortoise TG4/1998, which although did not show any intranuclear inclusions in the examined tissues was also considered conclusively infected by TeHV given that it was part of a previous challenge study carried out with the TeHV3 strain US1976/98. Ten out of 15 were *T. hermanni*, one *T. horsfieldii*, two *T. graeca*, one *Testudo* sp, one *Stigmochelys* (formerly *Geochelone*) *pardalis*. The tortoises died

either during the spring (April-June: n = 8) or the fall (October-December: n = 4) including also Z02/1970 that was euthanized because of very poor prognosis. One animal was part of a terminal transmission study and was euthanized in August. No information was available for two individuals. Seven were male and six female. No sex information was available for two tortoises. Most of the tortoises were from Switzerland (n = 11), fewer from Italy (n = 3) and one from the US, accounting for three countries and two continents. The selected cases spanned between 1998 and 2012. A complete summary concerning the tortoises' information is provided in Table 1.

Virus isolation

Cell cultures inoculated with tissue extracts from tortoise PN191/12 and swab washes from tortoise S12/1458 showed CPE consistent with cell rounding, detachment and cell lysis after 7 to 21 days and up to two blind passages performed. The presence of the virus in the supernatants of the cell cultures showing CPE was confirmed by PCR using the protocol described by Vandevanter and colleagues [33]. No virus could be isolated from the cell culture infected with the tissue extracts from tortoise PN186/12. Characterization of the TeHVs strains sequences DNA polymerase (DNApol). Partial amplification of the DNApol gene from all the tortoises included in the study (N = 15) was carried out with consensus primers according to an established protocol [33]. The PCR products were consistent with the expected size (181nt) and the DNA sequences were translated into the predicted 60 aa long sequence (58 aa readable for strain CH5132/08). Fourteen out of the fifteen sequences shared 100% identity with the homologous sequence of other TeHV3 strains (DQ343881; TeHV3 1976/96 = US1976/98). The remaining strain (IT13/08) showed 100% identity with the homologous sequence of TeHV1 strains (AB047545.1). The aligned sequences along with those of TeHVs reference strains are shown in Fig 3.DNApol aa sequence generated for the novel detected TeHV3 strains could not be deposited in the NCBI/Genbank database because their length was shorter than the minimum accepted.

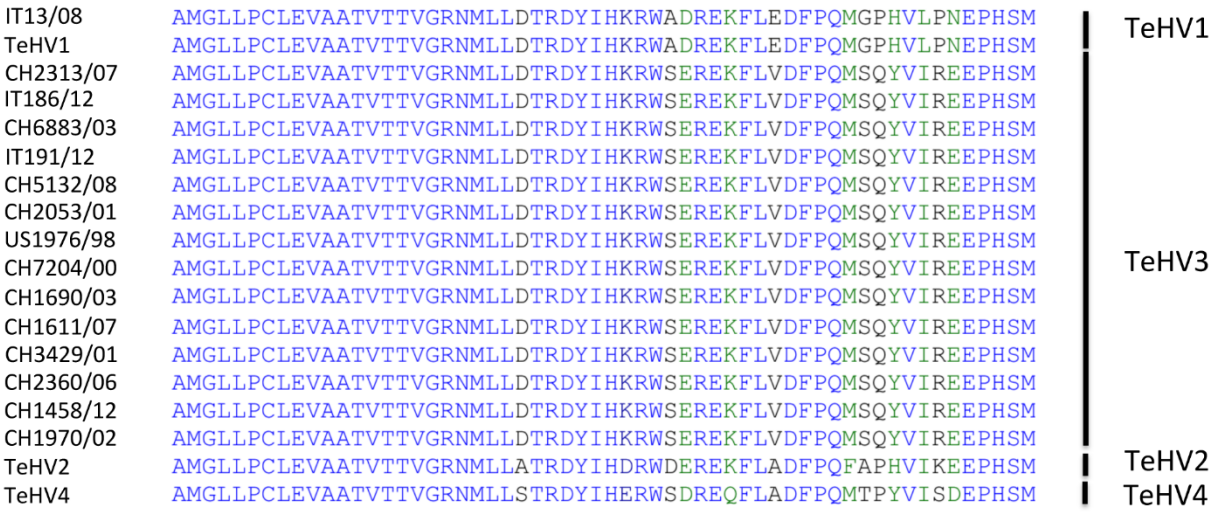

Figure 3: Alignment and comparison of the partial amino acid sequence of the DNA polymerase protein of the TeHVs strains. The alignment of the partial amino acid sequences of the DNA polymerase protein shows unique differences between the 4 recognized TeHVs genotypes. All but one of the detected TeHVs strains had sequences identical to TeHV3, whereas one strain belonged to genotype 1. (The Genbank accession numbers for TeHVs reference strains were: TeHV1-AB047545.1, TeHV2-AY916792.1, TeHV3 (1976/96 = US1976/98)-DQ343881, TeHV4-GQ222415.1. The partial DNA polymerase amino acid sequences of the TeHVs strains detected in this study could not be deposited in Genbank because their length was below the minimum required to be accepted).

Glycoprotein B (gB).

Within the portion of the genome identified as the UL region a 2,484 nt long ORF was determined to be the homologue of the HSV1 gB gene (UL27 = ORF52). The nucleotide sequence encoded for a protein 827 aa long. The protein was 25 aa shorter than the homologue of ChHV5 (852aa; Genbank AAU93326) and 38 aa shorter than that of ChHV6 viruses (865aa; Genbank AAM95776), the only reptilian herpesviruses with a complete available gB sequence.

Either complete or partial amplification of the gB gene was successfully obtained for all tortoises in the study (N = 15). In particular, the full amplification of the gB gene was obtained for strains US1976/98, IT191/12 and CH1458/12, and CH6883/03 (Table 1). The sequences obtained showed the highest variability in the 3'half of the gene. The 3' half partial sequence of the gB gene was then selected for phylogenetic analysis. The amplified portion of the gB gene was 1084 nt long (1069–1084 nt readable according to the amplicons, including the primers) and was obtained from all the remaining tortoises in the study (Table 1). The alignment of the complete and partial sequences of the gB genes of the different TeHVs strains revealed the existence of a total of 66 single nt polymorphisms (SNP) between two groups (genogroups) of strains (n = 11 and n = 3) named A and B, respectively. Two SNPs were located within the first 102 nt of the gB sequence, while the remaining 64 clustered in the 3'half of the gene. Furthermore, the strain CH3429/01 showed intermediate features between genogroup A and B and was identified as an additional putative group C. Of the 66 SNPs 33 were uniquely differentiating genogroup A from B (Fig 4). Five of the SNPs were missense, resulting in aa changes (Fig 5). All the missense SNPs clustered in the 3' half of the gene. Within a 250 nt-long region of the highly variable portion of the gB gene were clustered most of the SNPs (22 SNPs; 1,521–1,779 nt) differentiating genogroup A and B; similarly for the additional putative recombinant strain (genogroup C) (Fig 6). This region of the gB gene was then selected as target region for TeHV3 genotyping.

Genotype	Group	Nucleotide position																		
		102	1437	1449	1461	1482	1521	1524	1530	1536	1558	1569	1590	1608	1617	1620	1635	1641	1642
3	A	C	C	G	G	G	T	C	C	T	A	T	T	A	C	T	T	T	C
	B	A	A	A	A	A	A	A	G	C	G	G	C	C	T	C	C	C	T
	C	*	A	A	A	A	A	A	G	C	G	G	C	C	T	C	C	C	T

Genotype	Group	Nucleotide position														
		1671	1677	1695	1707	1711	1713	1722	1725	1779	1794	1821	1830	1836	1843	1932
3	A	C	A	G	G	C	G	C	G	C	A	T	A	A	G	G
	B	T	G	A	A	T	A	T	A	T	G	G	G	C	A	A
	C	C	A	G	G	C	G	C	G	C	A	T	A	A	G	G

Figure 4: Hypervariable region of the gB gene of TeHV3. The unique nucleotide changes unambiguously differentiating the genogroup A, B and C are shown in the figure (* = not available nucleotide for the putative group C TeHV strain).

Genotype	Group	Amino acid position				
		507	520	595	615	805
3	A	N	N	I	V	N/H
	B	K	D	I/V	I	H
	C	K	D	I	V	N

Figure 5: Hypervariable region of the TeHV3 gB protein. Three unambiguous amino acid changes differentiate the A and B genogroups within TeHV3 (colored blocks). The putative genogroup C shows an intermediate sequence between genogroup A and B.

Genotype	Group	Nucleotide position																
		102	1437	1449	1461	1482	1521	1524	1530	1536	1558	1569	1590	1608	1617	1620	1635	1641	1642
3	A	C	C	G	G	G	T	C	C	T	A	T	T	A	C	T	T	T	C
	B	A	A	A	A	A	A	A	G	C	G	G	C	C	T	C	C	C	T
	C	*	A	A	A	A	A	A	G	C	G	G	C	C	T	C	C	C	T

Genotype	Group	Nucleotide position														
		1671	1677	1695	1707	1711	1713	1722	1725	1779	1794	1821	1830	1836	1843	1932
3	A	C	A	G	G	C	G	C	G	C	A	T	A	A	G	G
	B	T	G	A	A	T	A	T	A	T	G	G	G	C	A	A
	C	C	A	G	G	C	G	C	G	C	A	T	A	A	G	G

Figure 6: Selection of hypervariable nucleotide changes for phylogenetic analysis. In a hypervariable region of the glycoprotein B gene spanning across approximately 250 nt, are clustered 22 of the 33 unambiguous nucleotide changes differentiating the A and B genogroups within TeHV3. This region was selected for a high-resolution phylogenetic analysis of TeHV3 strains. The light blue block highlights the selected region of the gene. (* = not available nucleotide for the putative group C TeHV strain).

The TeHV strain with intermediate SNPs between genogroup A and B (putative genogroup C) revealed a sharp regional demarcation of the SNPs arrangement. Briefly, the SNPs overlapping with those of genogroup A were located in the 3' end of the hypervariable region of the gB gene, whereas the SNPs overlapping with those of the genogroup B were located in the 5' end of the hypervariable region suggesting the occurrence of homologous recombination (Fig 7). The recombination event was confirmed by the software GENECONV, BootSCAN, MaxChi, Chimaera and 3Seq of the RDP4 package [51]. In particular, the MaxChi software identified the TeHV3 genogroup A as the most likely major parent and genogroup B as the most likely minor parent.

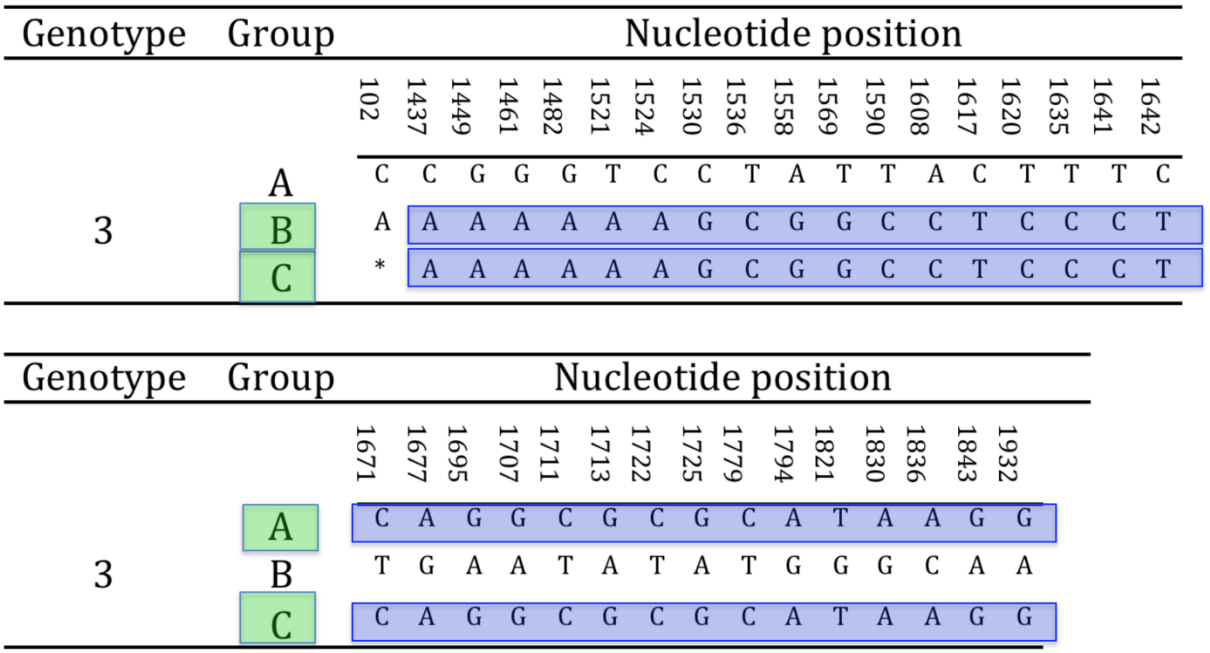

Figure 7: Glycoprotein B homologous recombination.

The putative C genogroup shows full identity with either genogroup A or B partial gB nt sequence according to the portion of the hypervariable region considered. This arrangement is consistent with the outcome of homologous recombination. The highlighted sequence blocks show the homologous regions between the gB sequences of B and C and A and B genogroups, respectively (* = not available nucleotide for the putative group C TeHV strain).

TeHV DNApol- and gB-based phylogenetic analyses (1, 2 and 3)

The phylogenetic analysis carried out on the partial aa sequences of the DNA pol (Type 1 analysis) revealed the presence of 14 strains clustering within TeHV3 and one within TeHV1 genotypes, respectively (Fig 8). In contrast, the phylogenetic analysis based on the partial aa

sequence of the gB protein (Type 1 analysis) revealed the existence of two distinct TeHV3 genogroups supported by significant bootstrap values (Fig 9). Additionally, the strain CH3429/01 clustered alone in an intermediate position between the two main TeHV3 genogroups paralleling the findings of the gB sequencing described above. The partial gB aa sequence of the single TeHV1 strain, IT13/08, clustered together with the TeHV3 genogroup A strains (Fig 9). Overlapping results were obtained independently from the alignment software used.

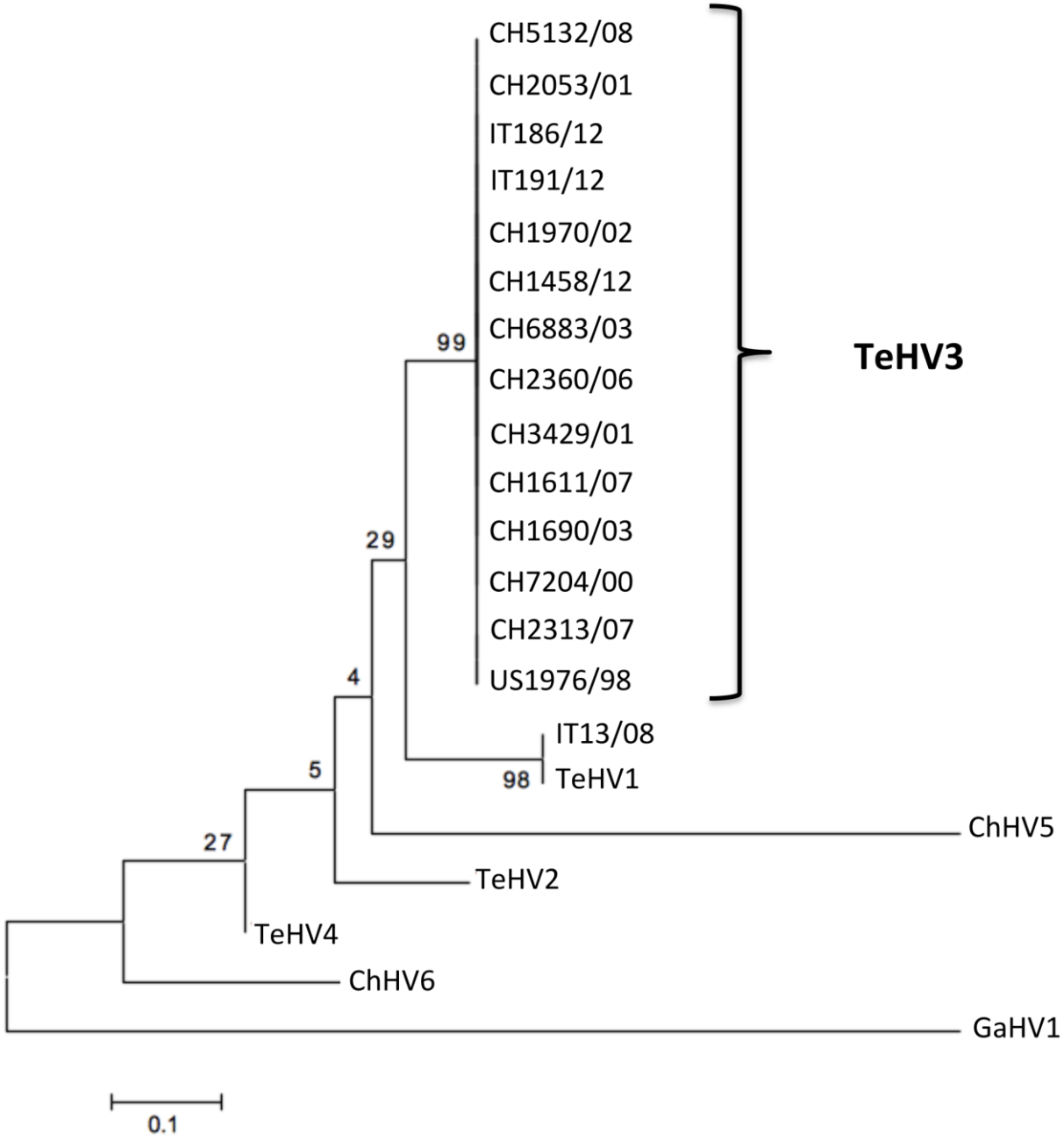

Figure 8: DNA polymerase-based phylogenetic analysis. Maximum likelihood tree inferred from the partial amino acid sequence of the DNA polymerase of the 15 investigated TeHVs strains and of the reference strains TeHV2, TeHV4, ChHV5, ChHV6 and Gallid herpesvirus 1

(GaHV1) that served as the outgroup. (Genbank accession numbers: TeHV1-AB047545.1, TeHV2-AY916792.1, TeHV3 (1976/96 = US1976/98) DQ343881, TeHV4-GQ222415.1, ChHV5-AF239684.2, ChHV6-EU006876.1, GaHV1- AF168792.1. No accession numbers could be obtained for the partial DNA polymerase protein sequences generated for the novel detected herpesvirus strains because their length was shorter than the minimum accepted by *Genbank).*

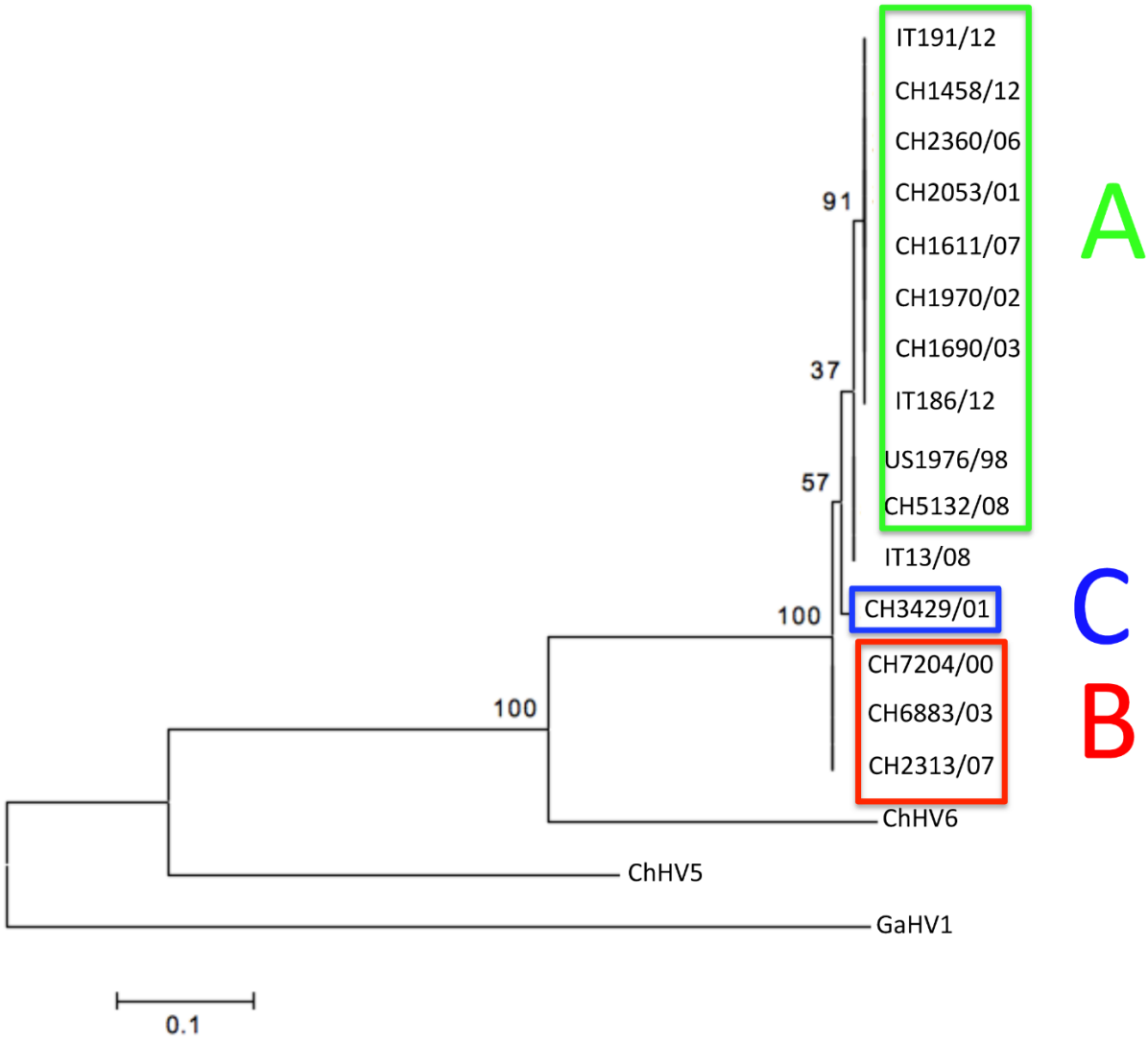

Figure 9: Glycoprotein B-based phylogenetic analysis. Maximum likelihood tree inferred from the partial aa sequence of the gB of the 15 TeHVs strains investigated in this study and of reference strains of other ChHV (ChHV5, -6). Gallid herpesvirus 1 (GaHV1) was included as outgroup. Bootstrap values from 500 iterations are shown. This analysis reveals the existence of at least two distinct genogroups within the TeHV3 genotype (A-green boxed and B-red boxed). A third putative genogroup (C-blue boxed) composed of a single strain is also observed. A TeHV1 strain (IT13/08) clusters within the A genogroup of the TeHV3 genotype.

Clear separation of the TeHV3 genogroups from the sequences of other two chelonian herpesviruses (ChHV5 and ChHV6) is observed. (Genbank accession numbers: CH1970/02-KP979727, CH1690/03-KP979726, CH1611/07-KP979721, CH2360/06-KP979725, CH2053/01-KP979723, CH5132/08-KP979729, CH7204/00-KP979719, CH6883/03-KP979730, CH2313/07-KP979722, CH3429/01-KP979720, CH1458/12-KP979718, US1976/98-KP979717, IT186/12-KP979728, IT191/12-KP979716, IT13/08-KP979724, ChHV5-AAU93326, ChHV6-AAM95776, GaHV1-YP_182356).

The separation between the genogroups A and B was even more robust when the phylogenetic analysis was carried out using the 250 nt of the hypervariable region of the gene described above (Type 2 analysis) (Fig 10).

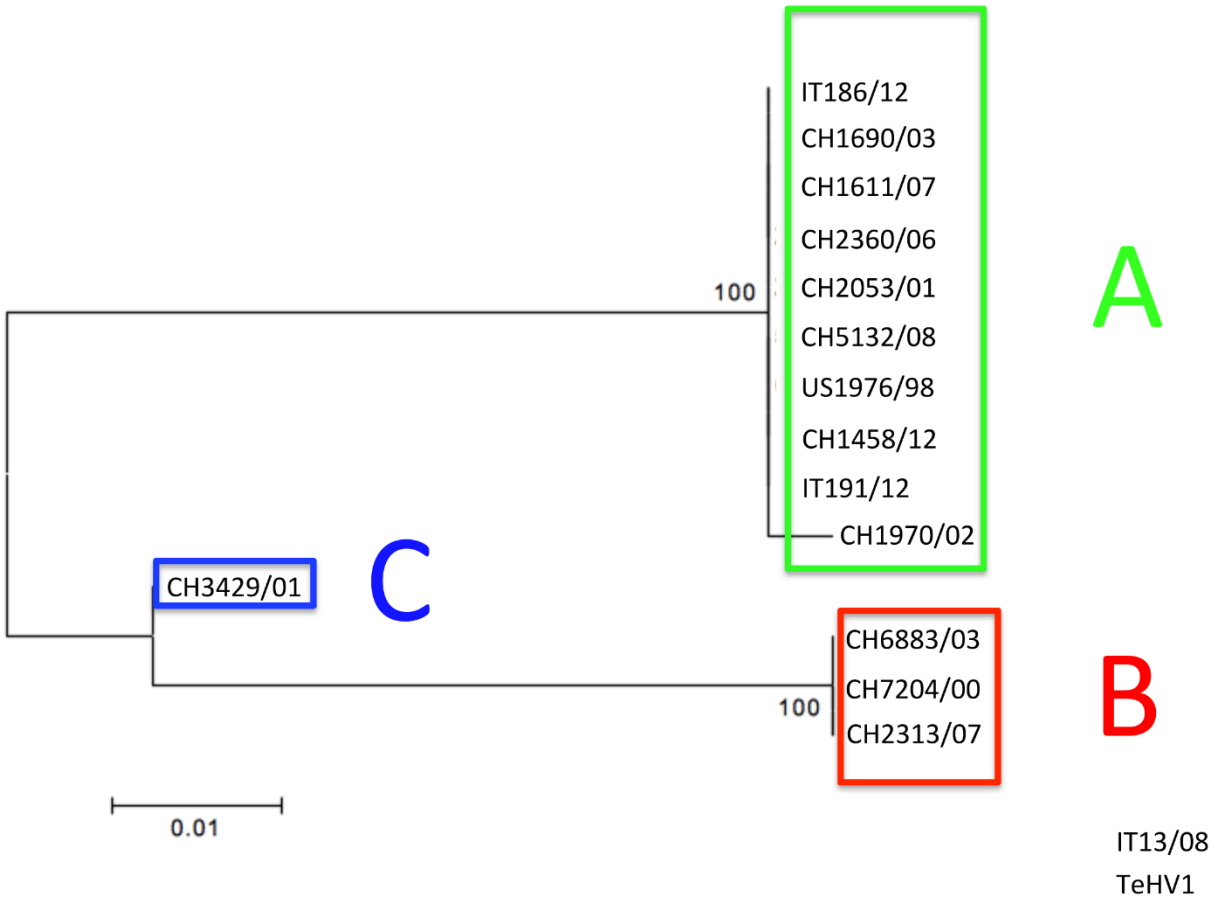

Fig 10: Glycoprotein B hypervariable region-based phylogenetic tree.

The phylogenetic analysis (Maximum likelihood tree) based on the hypervariable region of the glycoprotein B gene unambiguously identifies two main genogroups (A-green boxed and B-red boxed) and a third minor one (C) (blue boxed) within the TeHV3 genotype (Genbank accession numbers: CH1970/02-KP979727, CH1690/03-KP979726, CH1611/07-KP979721, CH2360/06-KP979725, CH2053/01-KP979723, CH5132/08-KP979729, CH7204/00-

KP979719, CH6883/03-KP979730, CH2313/07-KP979722, CH3429/01-KP979720, CH1458/12-KP979718, US1976/98-KP979717, IT186/12-KP979728, IT191/12-KP979716, IT13/08-KP979724).

Finally, when the full gB aa sequence was compared with the homologous sequence of other well-characterized herpesviruses, TeHV3 clustered unambiguously among the *Alphaherpesvirinae* in close association with ChHV5, the only recognized herpesviral species of the genus *Scutavirus* (Type 3 analysis) (Fig 11).

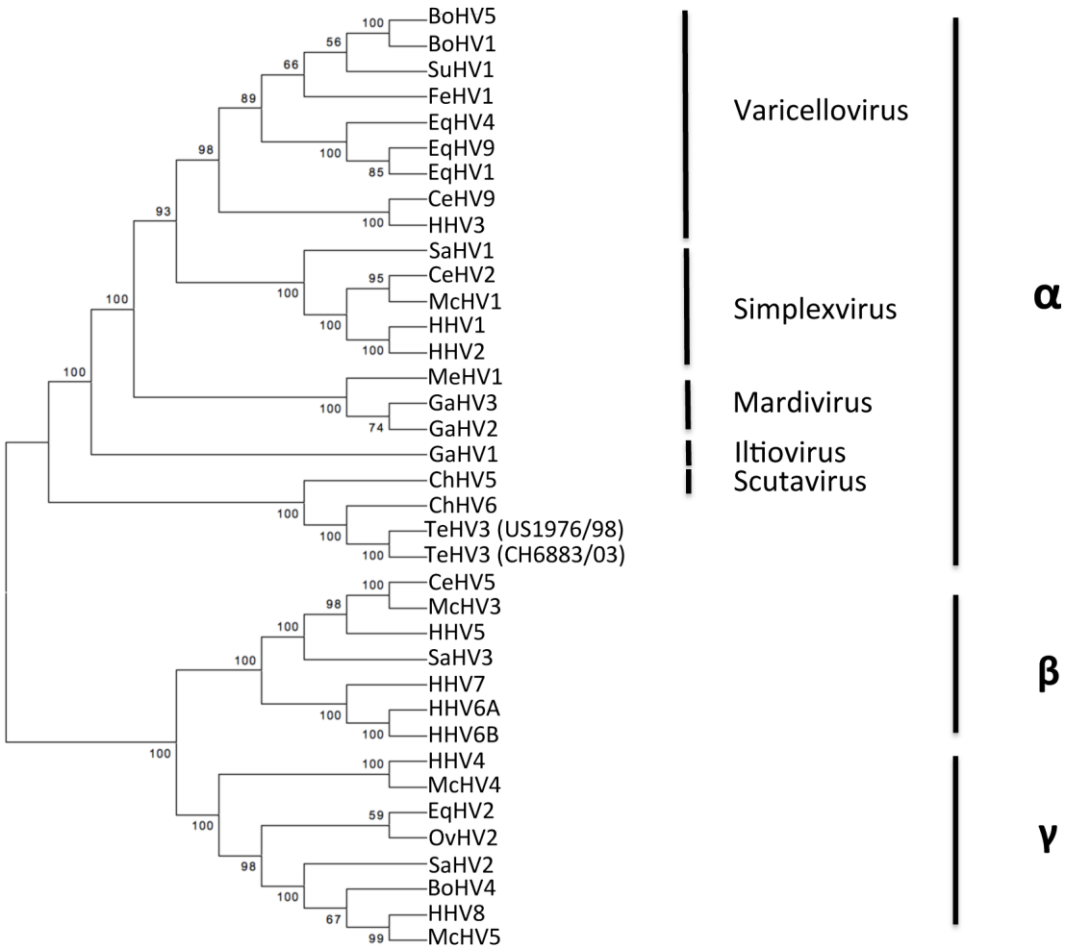

Fig 11: Glycoprotein B (full sequence)/based phylogenetic analysis. Maximum likelihood tree inferred from the full-length aa sequence of the gB of 2 strains of the TeHV3 and of 35 additional herpesviruses. Bootstrap values from 500 iterations are shown. This analysis shows unambiguous clustering of the TeHV3 main genogroups (A and B) within the Alphaherpesvirinae subfamily in close association with ChHV5 member of the novel identified genus of the *Scutavirus*. (Genbank accession numbers: Chelonian HV5-ChHV5-AAU93326, Chelonian HV6-ChHV5-AAM95776, Bovine HV5-BoHV5-YP_003662497.1, Bovine HV1-

BoHV1-NP_045331.1, Suid HV1-SuHV1-YP_068330.1, Feline HV1-FeHV1-YP_003331552.2, Equine HV4-EqHV4-NP_045250.1, Equine HV9-EqHV9-YP_002333514.1, Equine HV1-EqHV1-YP_053078.1, Cercopithecine HV9-CeHV9-NP_077446.1, Human HV3-HHV3-NP_040154.2, Saimirine HV1-SaHV1-YP_003933812.1, Cercopithecine HV2-CeHV2-YP_164470.1, Macacine HV1-McHV1-NP_851887.1, Human HV1-HHV1-NP_044629.1, Human HV2-HHV2-NP_044497.1, Meleagrid HV1-MeHV1-NP_073321.1, Gallid HV3-GaHV3-NP_066859.1, Gallid HV2-GaHV2-YP_001033956.1, Gallid HV1-GaHV1-YP_182356.1, Testudinid HV3-TeHV3-CH6883/03-KP979730, Testudinid HV3-TeHV3-US1976/98-KP979717; Cercopithecine HV5-CeHV5-YP_004936031.1, Macacine HV3-McHV3-YP_068182.1, Human HV5-HHV5-YP_081514.1, Saimirine HV3-SaHV3-YP_004940228.1, Human HV7-HHV7-YP_073779.1, Human HV6A-HHV6A-NP_042932.1, Human HV6B-HHV6B-NP_050220.1, Human HV4-HHV4-YP_001129508.1, Macacine HV4-McHV4-YP_068009.1, Equine HV2-EqHV2-NP_042604.1, Ovine HV2-OvHV2-YP_438135.1, Saimirine HV2-SaHV2-NP_040210.1, Bovine HV4-BoHV4-NP_076500.1, Human HV8-HHV8-YP_00119354.1, Macacine HV5-McHV5-NP_570749.1).

TeHV3 genogroups A and B appear to be associated with distinct lesional patterns

Pathological examination was performed on all the 15 tortoises in the study. A summary of the pathological findings is available in Table 1. Briefly, of all the tortoises infected with TeHV3 genogroup A strains (n = 10), four had inclusions limited to one tissue and no inclusions were detected in any tissue from the remaining tortoises infected with this TeHV3 genogroup. Differently, all the tortoises infected with TeHV3 genogroup B (n = 3) showed inclusions in at least one tissue and two tortoises had inclusions in more than one tissue. Pneumonia was observed both in tortoises infected with TeHV3 genogroups A and B, but it was overrepresented in the tortoises infected with TeHV3 genogroup B (two out of three). In contrast, in tortoises infected with TeHV3 genogroup A, pneumonia was seen in a minority of individuals (two out of eight with available lung tissue). Furthermore, inclusions were seen in the lung of both TeHV3-genogroup-B-infected tortoises with pneumonia, while no inclusions were seen in the affected lungs of tortoises infected with TeHV3 genogroup A. Of the tortoises infected with TeHV3 genogroup A for which brain tissue was available (n = 6) only one showed tissue changes consistent with meningitis. In contrast, meningitis was seen in two out of the three tortoises infected with TeHV3 genogroup B. Vasculitis and/or perivasculitis were observed in two out of three individuals infected with the TeHV3 genogroup B. The tortoise infected with the *bona fide* recombinant TeHV3 strain had inclusions in the tongue and

developed pneumonia. The only tortoise infected with a TeHV1 strain had pneumonia, tracheitis and hepatic lipidosis. Inclusions were seen only in the lung (Table 1).

Phylogeography of TeHV3 genogroups in Switzerland

The distribution of the infected tortoises from Switzerland was closely examined given that they were the most numerous and representative group. Fig 12 summarizes the locations of the tortoises infected with different TeHV3 strains. Tortoises infected with either genogroups A or B or the recombinant strain (C) were already present on the Swiss territory in 2000/2001 and additional cases of tortoises infected with either A or B genogroups were observed during the following years, at least up to 2008. In contrast, no additional infections with the recombinant TeHV3 strain were recorded (Fig 12).

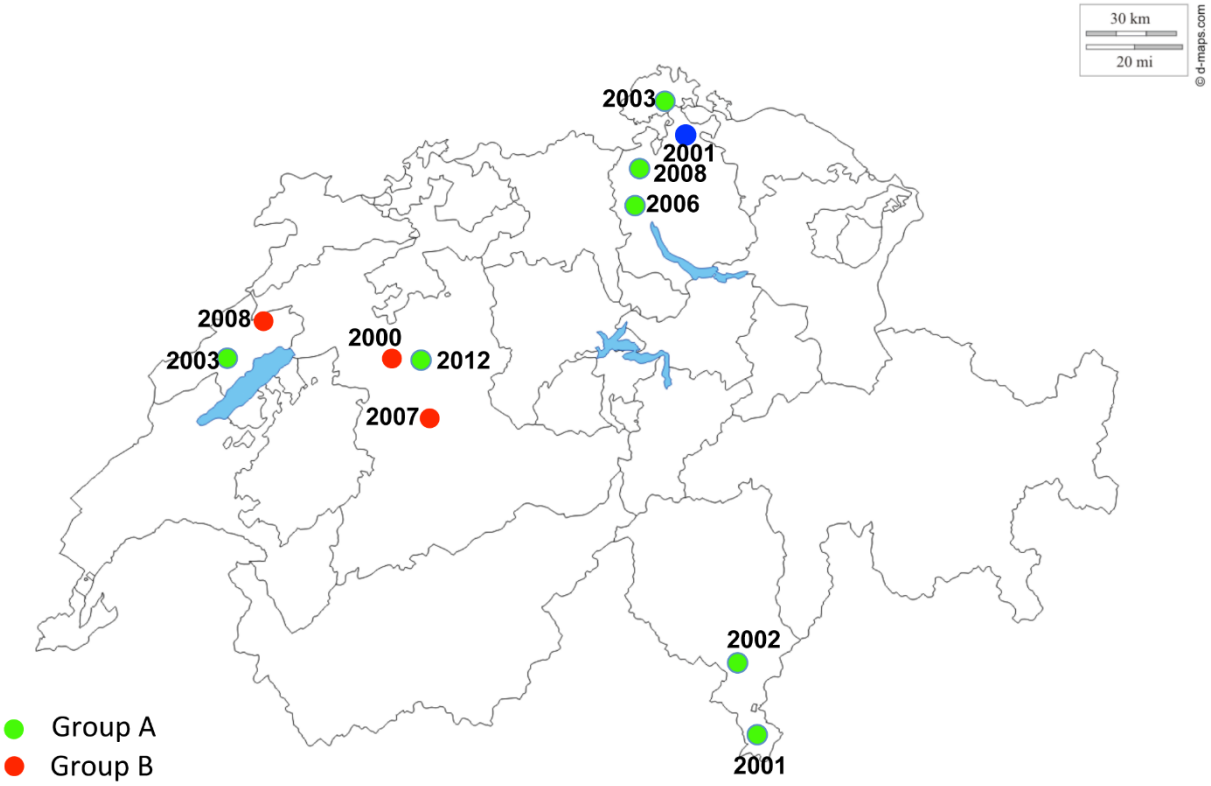

Fig 12: TeHV3 phylogeography (Switzerland). Green dots show the geographic origin of the tortoises infected with TeHV3 genogroup A, whereas red dots correspond to the geographic origin of the tortoises infected with TeHV3 genogroup B. The blue dot shows the location of the only tortoise infected with the recombinant strain originated from putative homologous recombination between a strain from the genogroup A and one from the genogroup B. The year next to the respective color dot correspond to the time when the specific tortoise died of TeHV3 infection. Main borders of Switzerland and of the Swiss cantons are also shown in the figure along with the main lakes (http://d-maps.com/carte.php?num_car=24787&lang=de).

Discussion:

The main goal of this research project was to fill a major gap concerning the biology of one of the most relevant viruses of reptiles, thereby providing new data to further investigate and better understand host-pathogen interactions in chelonians. This first example of a *de novo*assembly of a chelonian herpesvirus revealed that the TeHV3 genome shares several common features with other known *Alphaherpesvirinae* including length, gene-content and overall arrangement. The only other chelonian herpesvirus genome available to date is that of ChHV5. The two genomes share the overall arrangement and show the most similarities within the UL region. This is consistent with the findings of Alba et al. (2001) [55], with the most conserved gene blocks in the *Herpesviridae* spanning from the UL5 to the UL39 homologues. Despite the similarities between the two chelonian virus genomes, interesting differences were identified. For example, the two genomes appear to differ by approximately 18 Kb in length, with ChHV5 lacking several genes that were identified in TeHV3 [22]. The missing genes encode for proteins that apparently are dispensable for viral growth *in vitro* [22]. Among these are UL 46, 47, 48, 49.2, 49.5 and 51, which encode for structural elements such as tegument proteins. The absence of these genes might be compensated by other genes with similar functions that are present in the ChHV5 genome. However, other genes present in TeHV3 and absent in ChHV5 might have more critical roles for TeHV3. These include genes encoding for enzymes such as seronine-threonine kinase (UL13) that is likely relevant for intracellular signaling and cell cycling, and a dUTP diphosphatase (UL50), which together with the small subunit of the ribonucleotide reductase (UL40) and the thymidylate synthetase (UL45) are important for *de novo* synthesis of DNA. UL 54 encodes for the homologous gene of HSV1 ICP27 a potent gene transactivator.

Additionally, the lack of UL44 homologous gene encoding for the glycoprotein C (gC) in ChHV5 in comparison to TeHV3 might be critical for immune evasion. Glycoprotein C is known to bind to C3 and to inhibit virus neutralization [53].

The overall functional implications associated with the presence or absence of these genes in TeHV3 and ChHV5, respectively, are difficult to predict without the appropriate investigations. However, the lack of genes encoding for proteins relevant for the *de novo* synthesis of viral DNA and subsequently for viral replication, together with that of a major immediate early gene such as the homologue of ICP27 might help to explain some of the different features of TeHV3 and ChHV5, including for example the failure to grow ChHV5 in cell culture or the distinct pathological changes caused by these two viruses: necrotizing lesions in tor-

toises infected with TeHV3 and proliferative lesions of neoplastic nature in sea turtles infected with ChHV5. Further *in vitro* and *in vivo* studies are necessary to explore the functional bases for these differences. The differences in gene content of TeHV3 and ChHV5 may be secondary to differences in co-evolution that might have occurred with their respective hosts. Chelonians are a group of vertebrates that has evolved into multiple families that have diverged over millions of years [56], with adaptations to very different environments such as dry land and oceans. The genus *Testudo* comprises several species commonly infected by TeHV3. This genus is part of the family *Testudinidae*, which includes tortoises, dry land chelonians whose appearance in the fossil record dates back to the late Cretaceous [57]. Tortoises of the genus *Testudo* are considered to have originated in the African continent and later spread into Europe [56]. *Cheloniidae* is one of the two families of sea turtles having members susceptible to infection with ChHV5. Of the *Cheloniidae*, *Chelonia mydas* and *Caretta caretta* are two species of sea turtles most commonly infected by ChHV5. The presumptive long-standing independent co-evolution of the two viruses with hosts with distinct anatomical and physiological adaptations to their native habitats, may partially account for the different content in genes between TeHV3 and ChHV5. More sequencing data including that of TeHV2, the only North American TeHV genotype known to date are needed to contribute to better understand the actual correlation and evolutionary relationships between *Testudinid herpesviruses* and ChHV5. Finally, the presence in TeHV3 (and the absence in ChHV5) of the homologous gene of UL45, which encodes for the thymdylate synthetase gene present only in HHV3-VZV among the *Alphaherpesvirinae* but common in *Gammaherpesvirinae* was an interesting finding. However, despite this common feature with a *Varicellovirus*, TeHV3 does not appear to cluster in this genus. Furthermore, TeHV3 clusters closely to ChHV5, a *Scutavirus*. Their ancestors, the *Americhelydae* are believed to have originated in the North American continent during the Cretaceous [58], whereas their presence in the Mediterranean is considered to be more recent and occurred probably no more than 12,000 years ago [59]. No detection of ChHV5 has been yet reported in Mediterranean sea turtles. This suggests that it is unlikely that TeHV3 and ChHV5 might have diverged recently from a hypothetical common ancestor consistently with the results of the genome comparison (Fig 2). The novel genomic information described in this report is considered an important step to further illuminate the host-pathogen interactions in chelonians. The sequencing of the TeHV3 genome has allowed for the identification of several homologous genes to those of well-characterized herpesviruses.

Among these, the gB gene is one of the most critical genes for herpesvirus infectivity given the role of its encoded protein in cell entry [39, 40]. Furthermore, gB is both relatively well conserved among herpesviruses and at the same time it is under a likely higher evolutionary pressure than the DNApol because of the direct pressure exerted by the host immune system eliciting the production of neutralizing antibodies [40]. These features convinced us to select the gB gene sequence (and translated aa sequence) as an ideal phylogenetic marker to attempt to increase the phylogenetic resolution power among the TeHV3 strains and potentially trace correlations between distinct TeHV3 genogroups and pathology phenotypes in tortoises. The phylogenetic analysis performed with the partial sequence of gB, showed unambiguously the existence of at least two distinct genogroups of TeHV3 strains, named A and B, respectively, which could not be detected when using the partial DNA pol sequence. The phylogenetic analysis performed on a highly variable region of the gB gene further highlighted this distinction. The localization of four of the five missense substitutions spanning across a relatively central portion of the gene (1521-1845nt; Figs 5 and 6) is of interest given that it does not have a correspondence in the homologue gene of the type species of *Alphaherpesvirinae*, HSV1. Comparing different HSV1 gB aa sequences (data not shown), aa changes occur either within the very proximal N- and C-portion of the protein. The crystal structure of HSV1 gB has recently become available and antigenic and mutational analysis suggests that several domains spanning for most of the length of the protein ectodomain are required for virus entry [39]. The different clustering of aa changes occurring in TeHV3 gB might suggest a different arrangement of the most functionally relevant domains of TeHV3 gB than the HSV1's homologue. Similar distribution of the missense mutations is observed in ChHV5 strains. However, the specific functions of the different HSV1's gB domains are not yet fully understood and more investigations are needed to understand potential functional differences between HSV1's and TeHV3 gB.

Most of the identified strains in the fatally infected tortoises investigated in this study belonged to group A and a smaller number clustered within group B, possibly suggesting that subgroup A is the most common of the two genogroups. However, a large sample size is necessary to clarify this point.

A distinct third group (C), which was identified in a single tortoise, was shown to be the result of homologous recombination between members of the A and B genogroups consistent with co-infection of tortoises with both TeHV3 genogroups. To the best of our knowledge this is the first example of homologous recombination demonstrated in a chelonian herpesvirus and the first indirect example of multiple TeHV3 strain infection in tortoises. The gB-based phylogenetic analysis (either with aa or nt sequences) clustered the only detected TeHV1 strain together with TeHV3 genogroup A, suggesting that the phylogenetic relationships between distinct TeHVs might be more complex than previously considered and might be influenced also by homoplasy. Consequently, our novel genotyping method is complementary to the one based on the DNA pol, and we recommend for a more thorough and precise TeHVs characterization the following hierarchical approach: 1) identification of the TeHV genotype by sequencing of the partial DNA pol gene [33] and 2) identification of the TeHV3 genogroups based on the gB gene sequence.

Within the limitations associated with the small sample size, we observed differences between the pathology phenotype associated with TeHV3 genogroups A and B infection.

Specifically, TeHV3 genogroup B infection was associated with more frequent occurrence of intranuclear inclusions, viral pneumonia, vasculitis or perivasculitis and changes in the CNS than with TeHV3 genogroup A infection. These findings are suggesting of the existence of at least two subgroups of *Testudinid herpesvirus* responsible for different pathology.

The putative recombinant strain between A and B subgroups showed intermediate pathogenicity between the two. However, since this is based on a single isolate, further isolates need to be studied to confirm this observation. A transmission study carried out with both TeHV3 genogroups will be necessary to confirm the observations described above.

An important contribution of this investigation is the indirect assessment of some anecdotal observations reported by veterinarians and tortoise breeders; which include tortoises developing clinical signs of herpesvirus infection primarily in the spring or late fall. These observations support circumstantial reports from veterinarians and tortoise breeders that die-offs of tortoises secondary to TeHVs infection mostly occur after the end or just prior to hibernation. Furthermore, a presumptive different sensitivity of different species of tortoises to TeHV infection and/or disease has been suggested for the Mediterranean Hermann's and Greek tortoise [14].

Consistently, Hermann's tortoises were overrepresented in our study group suggesting that this species is more sensitive to TeHV3. Further work is needed to substantiate these findings.

In conclusion, we have successfully performed the first complete *de novo* assembly of a chelonian herpesvirus, providing fundamental genetic information to obtain greater insight into the biology of this virus and the host-pathogen interaction in an early diverging vertebrate lineage. This enabled us to identify at least two distinct subgroups of the TeHV3 genotype and the distinct lesional profiles caused by these viruses in the tortoises examined in this study. This unearths new foundations for future studies on host-parasite resistance and infection.

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FIRST DETECTION OF TWO DISTINCT LINEAGES OF TESTUDININD HERPESVIRUS 3 (TeHV-3) IN MEDITERRANEAN TORTOISES

F.C. Origgi,[1]* M. Tecilla,[2] P. Pilo,[3] G. Grilli,[2] and P. Roccabianca[2]

[1]Centre for Fish and Wildlife Health (FIWI), Vetsuisse Faculty, University of Bern, Switzerland; [2]Dipartimento di Patologia Animale, Igiene e Sanita' Pubblica Veterinaria, Universita' di Milano, Italy; [3]IVB, Vetsuisse Faculty, University of Bern, Switzerland

ABSTRACT

Testudinid herpesviruses (TeHVs) are among the most relevant viral pathogens of tortoises. Molecular and serologic tests have greatly contributed to define the phylogentic and antigenic relationships between the four different genotypes known up to date. Of these, TeHV-3 is globally distributed and it is considered the most virulent. Molecular genotyping of TeHVs has been carried out on the basis of the molecular differences present in a 181 base-pair long, very conserved region of the DNA polymerase (DNApol), a similarly very conserved gene among herpesviruses. The great stability of this genomic region has the intrinsic limit of a poor phylogenetic resolution beyond genotypes. In order to tackle this problem we sequenced for the first time the entire glycoprotein B (gB) gene of TeHV-3, which is also well conserved among herpesviruses, but under a higher evolutionary pressure than the DNApol resulting in more frequent mutations. We selected 15 TeHV-3 strains either isolated and/or detected in fatally-infected Mediterranean tortoises and for each of them we performed either the complete or partial sequence of gB along with the partial sequence of their DNA polymerase genes. The derived amino acid sequences were aligned with five different software types (ClustalW, ClustalW2, T-Coffee, MUSCLE, MAFFT) and phylogenetic trees were resolved by Maximum-likelihood. Results showed that there are at least two distinct groups of viruses that can be differentiated on the basis of their gB but not on that of their DNApol sequences, respectively, consistent with the existence of at least two distinct lineages within the TeHV-3 genotype.

Chapter 7: *Testudo graeca* immune response against *Testudinid herpesvirus* 3

Initial assessment

Original title: **TeHV3 glycoprotein B: shedding light on host-pathogen interaction in chelonians.**

Authors**:** Marco Tecilla[a], Paola Roccabianca[a], Paola Pilo[b], Francesco Origgi[b,c].

[a] Department of Veterinary Medicine (DiMeVet), School of Veterinary Medicine, University of Milan, 20010, Milan, Italy

[b] Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Laenggassstrasse 122, 3012, Bern, Switzerland.

[c] Centre for Fish and Wildlife Health (FIWI), Vetsuisse Faculty, University of Bern, Laenggassstrasse 122, 3012, Bern, Switzerland.

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Numerous infectious diseases have been documented in reptiles, however scant information is available concerning their associated immunological response. One of the most diffuse and lethal reptile pathogens is *Testudinid herpesvirus* 3 (TeHV3), a member of the subfamily Alpha-herpesvirinae. All species of tortoises (*Testudinide*) are considered susceptible to TeHV3, however the virus is overrepresented in the genus *Testudo*, which includes, among others, *T. graeca*, *T. hermanni*, *T. marginata*, and *T. horsfieldii*, which are popular pets in Europe. The occurrence of TeHV3-associated diseases is highest right after hibernation.

The aim of this work is to partially characterize the immunological response to *T. graeca* against TeHV3. A bacteriophage library composed of about 5,000 clones containing genomic DNA fragments of TeHV3 was produced. Bacteriophages were amplified and were screened with TeHV3-seropositive sera from *T. graeca*. Phagemids were excised from the positive bacteriophages, sequenced, and compared with the TeHV3 genome to identify the encoding genes. Six different structural and non-structural proteins were initially identified as immune relevant. Phagemids of the positive clones were transfected into Vero cells, and the expression of the encoded polypeptides was assessed by FACS using *T. graeca* seropositive sera. From all six selected clones, only that expressing a partial sequence of the glycoprotein B (gB) showed an unambiguous positive signal in the FACS analysis. This result is consistent with the well-known immunogenicity of gB of other herpesviruses, including those infecting humans, and with the remarkable highly conserved role of gB in host-pathogen interaction across species and evolution.

Introduction:

More than 130 herpesvirales have been documented to infect animals [1,2], and from them, at least 30 (~39%) have been detected in reptiles. At least one herpesvirus has been detected in each of the reptilian orders, with the only exception of Ryncocephalia [3]. Herpesvirus infections in reptiles may be associated with either clinically undetectable forms [4,5] or with obvious diseases including neoplasia [6–8]. However, the most frequently associated findings with herpesvirus infections in reptiles are necrotizing [3,9,10] lesions.

The classification of chelonian herpesvirus comprises different groups, including that of *Chelonid herpesvirus*es [11–15] (ChHVs), [6,16–19] *Testudinid herpesvirus* (TeHVs), [3,4,9,10,15,20–26] *Emydid herpesvirus* (EmyHVs), [12,27] and Terrapene herpesvirus (TerHVs) [8,28]. It is likely that the current taxonomy of chelonian herpesvirus will undergo extensive revision in light of the new genetic information currently accumulating in the available databases [3,4,6,11,14,29–32].

Testudind herpesviruses (TeHVs) include four distinct genotypes detected only in tortoises. Two of them, genotype 1 and 3, are considered of Eurasian origin and are currently the most diffuse and overrepresented in the genus *Testudo spp.* [3,9,10,21,23,24,30,31,42–45]. From the other two genotypes, TeHV2 has been detected only in the US, whereas TeHV4 is the first detected African genotype [3] and has been reported in an asymptomatic bowsprit tortoise (*Chersina angulata*) [4] and just recently in a leopard tortoise (*Stigmochelys pardalis*) with respiratory signs. [46]

Of all TeHVs genotypes, TeHV3 has been associated with high mortality and morbidity in several species of tortoises, with *Testudo hermanni* being the most sensitive and *T. graeca* the most resistant to the viral disease [3,47–49]. Infected animals usually develop nasal discharge, mono or bilateral conjunctivitis, pneumonia and diphteronecrotic stomatitis and/or glossitis that, in most severe cases, can extend aborally involving esophagus and trachea. The histological hallmark of herpesvirus infection is the presence of intranuclear eosinophilic/amphophilic inclusion bodies in epithelial cells of various organ systems and/or in neurons [3,46–48]. Recently the entire TeHV3 genome has been sequenced, revealing the existence of at least two genogroups of this virus presumably associated with distinct pathology [14]. Furthermore, Gandar et al. [53] demonstrate the presence of three continuous genomic unique regions (U_T, U_L and U_S), two of which (U_T and U_S) are flanked by inverted repeats. [53] The presence of inverted repeats near the two unique sequences suggests that TeHV3 may have four genome isomers differing by the orientation of U_T and U_L.

Despite the extensive work carried out on TeHV3 in the last 20 years, the limited knowledge available on reptiles' immune system has restricted the possibilities to understand the dynamic of the infection and the mechanisms behind the host-pathogen interaction in this animal group [54–62]. For better understating of reptiles' immune system, it is not only necessary to fill the gap in comparative immunology but also to implement immunological therapies including vaccinations.

The aim of our work was to investigate the tortoises' antiviral immune response by evaluating the antigenicity of the whole viral proteome, using TeHV3 as model.

To investigate the TeHV3 antigenic proteins we built a phage expression library starting from the whole genome of the TeHV3 type strain US1978/98.

Phage display libraries have been widely used to investigate the host-pathogen interaction in human medicine. This technique allows to insert fragments of genomic DNA into bacteriophages that express the encoded proteins. [63]

The library was screened with hyper-immune sera obtained from Greek tortoises infected with TeHV3 during a transmission study. [64] Here we show how this approach allowed the detection of a highly immunogenic viral protein, which could be considered for the development of future antiviral treatments in Chelonians including that of a vaccine.

Materials and methods:

Virus and genomic DNA: The TeHV3 strain US1978/98 [9] *was grown on Terrapene* Heart Cells *(TH-1; ATCC-CCL 50 Sub-line B1; American Type Culture Collection, Rockville, MD, USA) according to an established protocol* [14,25,65]. *The TeHV3 genomic DNA was obtained from the same strain similarly to what was previously described* [14,25].

Phage Library and DNA sequencing:

Library preparation: The DNA fragments to insert into the bacteriophage were obtained according to the manufacturer's instructions. Briefly, distinct batches of US1978/98 genomic DNA were fragmented by restriction digestion with Sao3AI for different lengths of time. The digested DNA batches were then resolved in a 1% DNA agarose gel. The digested batch whose TeHV3 genomic DNA fragments ranged between 0.2 to 2 Kb was selected and the fragments were ligated into the bacteriophage genome using an auto-assembly bacteriophage kit (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US).

Phage amplification: The library was amplified according to the manufacturer's instructions (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US). Briefly, lambda bacteriophages containing TeHV3 genomic fragments were initially expanded infecting the E. coli

bacterial strain XL1- MRF (ZAP Express EcoR I/Xho I Library, Agilent Technologie, US). Bacteria were incubated with the bacteriophage suspension for six hours at 37°C while shaking. Subsequently the suspension containing both bacteria and bacteriophages was mixed with LB-top-agar and overlaid on LB-agar in Petri dishes and incubated overnight at 37°C. The plates showing plaques of lysis were then overlaid with 8ml of SM buffer to harvest the bacteriophages. The buffer was subsequently recovered and stored at +4C. After that, the bacteriophage suspension was titrated by ten-fold dilutions, using the same procedure as described above to determine the optimal concentration of the phages to infect the bacteria.

Sera collection: TeHV3 seropositive and seronegative sera samples were obtained from Greek tortoises (*Testudo graeca*) that were experimentally infected with either one of two strains of TeHV3 (US1978/98 and D4295/75) [65] or from spontaneously infected *T. graeca*.

Library screening: Two consecutive screenings were performed on the bacteriophage library. In both XL1-MRF *E. coli* were infected with the selected suspension of bacteriophage as described above and incubated at 37°C overnight. An estimate of the number of clones carrying a fragment of the TeHV3 genome was made by white/blue selection (Figure 1) carried out according to the standard protocol. [65]

Plates were then cooled for 1hr at +4° and drawn to properly orientate the membrane for the selection of the positive clones.

Membranes were then immersed in blocking solution (TBS-T with 5% skimmed milk powder) for 1hr at room temperature on a rocker. After that they were washed twice with blocking solution for 5 minutes each, and twice with PBS for 30 seconds each. In the first screening the membranes were incubated with hyper-immune tortoise sera from the first transmission study performed on TeHV3 by Origgi et al. [65]. During the second screening, the membranes were incubated in a mix of tortoise sera, composed of 50% hyper-immune serum from the transmission study and 50% serum from ELISA positive, spontaneously infected tortoises. Sera were diluted 1:20 in the blocking solution for 1hr and washed as described above. A biotinylated mouse monoclonal antibody (HL1546) directed against the heavy chain of *Testudo* spp. IgY was used as secondary antibody [25] at the concentration of 1µg/ml in PBS. Membranes were incubated with the primary antibody for 1hr on a rocker at room temperature and then washed as described above. A mixture of 2 µl AP-Streptavidin (Zymed, 43-4322) and 2 µl of polyclonal antibody rabbit anti-mouse (Jackson ImmunoResearch, 315-005-003) diluted in 1ml of PBS was then applied to membranes for 1 hour at room temperature. Following five washes of 10 seconds each with distilled water, membranes were finally stained with the chromogen substrate composed of 45 µl NBT (18.5 mg/ml nitro blue tetrazolium chloride)

with 35 µl BCIP (9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate) diluted in 10ml of SM-Buffer (10ml 1M carbonate buffer + 10 ml 100mM MgCl2 + 980ml H2O). The chromogenic reaction was closely monitored for three minutes and then the membranes were rinsed 4-5 times with distilled water and immediately dried with Whatman 3M paper (Sigma-Aldrich, US).

Positive bacteriophages were punched out from the plate using a 20µl tip. These phages were amplified and stored at 4°C according to the manufacturer's instructions (ZAP Express Predigested Vector Kit and ZAP Express Gigapack Cloning Kits, Agilent Technologies).

The positive clones from the primary screening were then expanded and tested again with hyper-immune tortoise sera similarly to what is described above. Following this, the selected clones were amplified in E. coli strain XLOLR to obtain the phagemids as described by the manufacturer (ZAP Express Predigested Vector Kit and ZAP Express Gigapack Cloning Kits, Agilent Technologies). Phagemids were then digested with SAO3AI to obtain the correspondent TeHV3 genomic inserts and resolved in a 2% agarose gel.

Sequencing: Each of the positive clones was then partially sequenced in order to determine the identity of the genomic insert. A mastermix was composed of 3µl of 10x PCR Buffer (Qiagen, Silicon Valley, USA), 0.4µl of dMTs, 2µl each of FW and RW Turboprimers (TriLink, San Diego, USA), 0.1µl of Taq (Qiagen, Silicon Valley, USA), and 21.5µl of DNA-RAN free water. Samples were then placed in a thermocycler (Thermofisher Inc., US) and amplified for the first time. The amplification program consisted of 3 minutes' denaturation at 94°C, then 35x amplification cycles subdivided in 30 sec. at 94°C denaturation, 30 sec. at 65°C annealing and 2 minutes at 72°C elongation, with a final extension of 10 minutes 72°C to exhaust the polymerase. Each PCR amplicon was submitted for automated Sanger sequencing (ABI Prism 3700, Applied Biosystems) using the Big-dye terminator technology. Briefly, a master-mix containing 2µl of Sequencing Buffer 5x (Thermofisher Inc., US), 0.5µl of either FW or RV Primer (10µM), 2µl of BigDye (Thermofisher Inc., US), 2 µl of DNA and ddH_2O up to 10 µl was prepared. Sequencing was carried out using the same primers used for PCR amplification as described above.

Fluorescence Activated Cells Sorting (FACS) Validation in VERO cells:

Eukaryotic cell transformation: Extracted phagemid clones were used to transfect VERO cells. Briefly, 10^5 Vero cells were seeded in each well of a 6-well plate (TPP, Trasadingen, Switzerland) on the day prior to transfection.

The following day, one microgram of phagemid was mixed with 135µl of OptiMEM (Thermoscientifc, US) and 3µl of TransIT-LT1 (Mirus Bio LLC, Medison WI, US) in Eppendorf tubes. A single well was used for each of the tested phagemids. Tubes were incubated for 15 minutes at room temperature. The suspension was then added to each well. Plates were gently mixed and incubated overnight at 37°C. Each phagemid was transfected in duplicate.
Cell membrane fixation and permeabilization: One of the duplicates underwent permeabilization, while the other did not. Initially, the cell monolayers of each well were fixed with cold (4°C) 10% buffered formalin for 5 minutes, directly into the well. Later, cells were chemically and mechanically detached by using PBS 0.5 mM EDTA incubated for 30 minutes at 37°C and sterile cells scrapers (TPP, Switzerland), respectively. Detached cells were collected in 10 ml Falcon tubes (TPP, Switzerland) and centrifuged at 300g for 15 minutes. After two PBS washes, cells were resuspended in 10ml PBS. The cells that underwent permeabilization were instead resuspended into a 0.2% Tween 20 PBS solution, and incubated at 37°C for 15 minutes. After permeabilization cells were pelleted at 300g for 5 minutes, and finally resuspended in PBS.

Cellular staining: The same staining procedure was performed for both permeabilized and not permeabilized cells to evaluate the expression of intracellular and superficial proteins. Cell tubes were centrifuged at 300g for 15 minutes and resuspended in to 300µl of DPBS with 15µl of seropositive *T. graeca* serum (1/20). Tubes were incubated at +4C° for one and a half hours. After incubation, cells were centrifuged (300g x 15min), washed two times with DPBS, and then resuspended in 1ml DPBS with 0.1µl of primary antibody (HL1546 mouse anti turtle-IgY), and incubated for 1 hour at room temperature. The primary antibody was washed away as described above.
Later, a solution of 1ml DPBS with 0.5µl of secondary antibody (rabbit anti mouse, Jackson ImmunoResearch, 315-005-003) was incubated for 1 hour at 4°C with the cells. Following an additional wash, cells were resuspended in a solution of 1ml DPBS with 1µl of a green fluorochrome anti rabbit (Alexia fluor 488, Thermofisher, US). Cells were incubated with a green fluorochrome anti rabbit (Alexia fluor 488, Thermofisher, US) for 1.30 hours and then centrifugated (300g x 15 min), washed three times with DPBS, and resuspended in PBS.

Results:

Bacteriophage library screening and clone sequencing:

The optimal working dilution for the bacteriophage suspension was determined to be 10^{-3}.

The initial screening of the phage library carried out with hyperimmune sera obtained by experimentally infected Greek tortoises [64] revealed 16 positive out of approximately 5,000 clones, accounting for 0.32%.

A second screening carried out on each of the amplified clones confirmed the positivity of clone 1 and 6 (12.5%), whereas all the other clones were negative and were considered as false positives.

Clone 1 contained a 1.8 Kb long DNA fragment corresponding to the entire gene of the ribonucleotide reductase and to a partial sequence of the major capsid protein encoding gene of TeHV3. Clone 6 contained a 2.3 Kb long DNA fragment corresponding to the partial sequence of the glycoprotein B encoding gene.

The initially positive 16 clones were screened a second time by using a mix of both experimentally and spontaneously infected Greek tortoise sera. Among the 16 tortoises, 6 tested positive (37.5%), but only 5 carried a DNA insert (Figure 2).

Clone 1a contained 2 Kb of DNA containing part of the large sub-unit of ribonucleotide reductase and a fragment of the tegument protein genes. Clone 1b contained 2 Kb of DNA encoding for a partial sequence of the glycoprotein B gene. Clone 3 contained 500 bp of DNA encoding for part of the envelope protein gene. Clone 6 contained 300 bp of DNA encoding for a fragment of the immediate early 2 gene. Clone 7 contained 600 bp of DNA encoding for a part of the uracil DNA glycosylase gene (Figure 1).

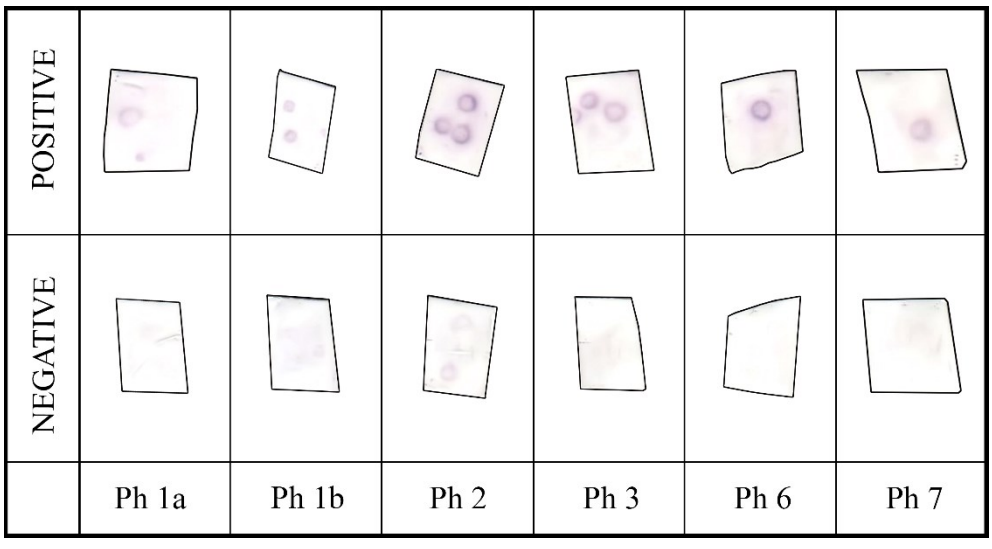

Figure 1: Immunoblot nitrocellulose membranes of the six selected clones. For each clone, positive sample and negative controls are shown. The criteria used to determine if the reaction

was a true or a false positive was the presence of a marked difference between the reaction intensity, and stain size and definition compared with the negative control. Clone 2, which shown a strong immunoblot positivity, was then empty at sequencing.

Eukaryotic cell transformation and FACS:

The clones that were confirmed positive during the second screening were then selected for expression in Vero cells. Expression of the partial sequences of the genes carried by the positive phagemids was assessed by FACS analysis (Figure 2*).*

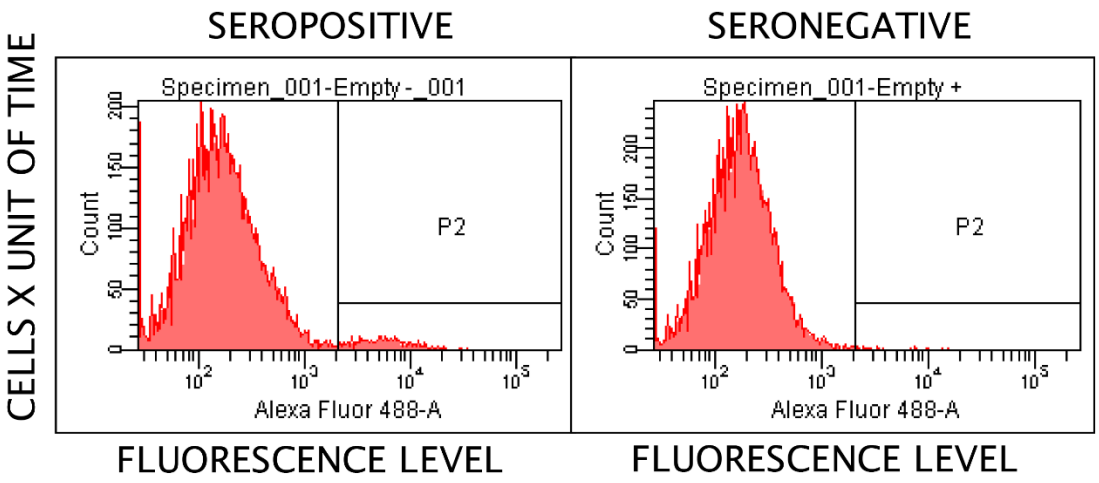

Figure 2: Fluorescence level detected via FASC in gB sample (left) and in gB negative control (right). Although the difference is minimal, all the other positive samples showed a fluorescence pattern similar to the detected for negative control, making this a significant difference.

We considered that in order to validate the selected clones, it was necessary to test them in a different setting to that used initially, in order to provide an independent second line of evidence for our results.

Discussion:

This work aimed to start evaluating the host-pathogen interaction between *Testudo graeca* and TeHV3 by investigating the most relevant immunogenic proteins of this virus.

The viral proteins were expressed in bacteria using a bacteriophage display library, and serologically screened using hyperimmune *Testudo graeca* sera obtained from a transmission study [64]. Phage library is a molecular technique widely used to investigate infectious diseases in human beings [66] and occasionally in veterinary medicine [67,68]. Phage displays have been used with pathogens of different mammals [67], birds [68] and fish [69] species, but this was the very first time they were applied to reptiles. The most relevant result obtained was the identification of the glycoprotein B as an immunogenic protein in TeHV3.

Among the herpesviruses, the most antigenic proteins identified both in human beings and animals [29,70,71] are the major tegument protein (UL37) and the family of the glycoproteins [72]. Glycoproteins are a class of capsidic proteins involved in the attachment and entry of viral particles into the host cell [73]. Three major classes of glycoproteins have been identified (B, D and G) and all of them seem to have a crucial role in the infection of the host cell by the virus [74–76]. Among the glycoproteins, gB is considered a relevant and highly conserved immunogenic molecule in herpesvirales [77,78]. Our results also confirm the role of this protein in a very ancient herpesvirus like TeHV3, as previously suggested by Origgi et al. [14] Furthermore, the identification of gB can be considered an internal control, demonstrating the effectiveness of the phage display library in reptiles. The immunological role of gB, along with its genetic structure, shed light on the phylogenetical origin of the herpesviruses and their evolution. The key role of gB in the immune response of *Testudo graeca* against TeHV3 can have both diagnostic and clinical implications. The discovery of the immunological role of gB, in association with knowledge of the gB sequence, open the door to develop new and more specific tests, and potentially to vaccination. Different diagnostic tests have been developed to detect TeHV3 in living tortoises [3], among which ELISA is the most commonly used. The actual ELISA protocol to detect TeHV3 [25] has been demonstrated to cross react with TeHV2 [21], thus not providing a sufficient specificity level. Furthermore, recent studies on the TeHV3 genome have demonstrated the presence of at least two subtypes of gB correlating with different level of lesion severity [14]. New diagnostic tests based on the recognition of gB isotypes will more specific and will provide more clinical information. The chance to clone gB might also be used to develop a vaccination, protecting the animal from the infection or reducing the clinical signs of the disease. Even if the use of gB based vaccinations have failed to protect human beings, they might protect against more ancient herpesviruses

with a potentially lower recombination rate compared to the human herpes simplex or cytomegalovirus, against which gB based vaccinations have been tested [77–79]. However, there is no information available about the possible response of reptiles to vaccination.

During this experiment, other five proteins were serologically detected as immunorelevant, but were negative to the FACS.

The expression of the selected clones in a eukaryotic system implies the glycosylation of the residues to the candidate immunogenic peptides, which differs to what occurs in prokaryotic systems. Glycosylation was expected to closely reproduce the native state of the viral peptide, providing more informative data concerning the actual antigenicity of the candidate peptides. Transfected cells were tested either with or without cell membrane permeabilization. Unambiguous positivity was confirmed by FASCS analysis only for the surface expression of clone 1b, encoding for the partial sequence of the gB gene (Figure 3). No other clones showed convincing positive signals, despite cell permeabilization, increased amounts of transfected DNA phagemids, prolonged expression time of the transfected cells, or the use of other fluorescent dyes.

The expression of the proteins in eukaryotic cells may have led to conformation changes not allowing allostery binding of the antibody to the protein, with consequent misdetection. However, the possibility of false positives has to be considered, and the proteins should be retested using a different technique.

This is the very first study evaluating the immunological response of reptiles against viruses and the host-pathogen interaction. As previously demonstrated in other herpesviruses, the gB represents an immunologically important antigen, based on which new diagnostic tools and vaccinations can be developed.

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CORRESPONDING AUTHOR

Marco Tecilla
marco.tecilla@unimi.it

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Characterization of the immune response against Testudinid herpesvirus 3.

M. Tecilla[a*], P. Roccabianca[a], P. Pilo[b], F. Origgi[c]

[a] *Department of Veterinary Science and Public Health (DIVET), School of Veterinary Medicine, University of Milan, 20010, Milan, Italy.*

[b] *Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Laenggassstrasse 122, 3012, Bern, Switzerland.*

[c] *Centre for Fish and Wildlife Health (FIWI), Vetsuisse Faculty, University of Bern, Laenggassstrasse 122, 3012, Bern, Switzerland.*

Abstract

Numerous infectious diseases have been documented in reptiles, however minimal information is available concerning their immunological response. One of the most diffuse and lethal reptile pathogen is *Testudinid herpesvirus* 3 (TeHV3), a *Alphaherpesvirinae*. All species of tortoises (*Testudinide*) are considered susceptible to TeHV3, however the virus is over represented in the genus *Testudo*, which includes, among others, T. *graeca*, T. *hermanni*, T. *marginata*, and T. *horsfieldii*, that are popular pets in Europe. Incidence of TeHV3-associated disease is highest right after hibernation (Origgi, 2012).
The aim of this work is to partially characterize the immunological response of T. *graeca* against TeHV3. A bacteriophage library composed of about 5.000 clones containing genomic DNA fragments of TeHV3 was produced. Bacteriophages were amplified in a specific strain of E. *coli* and were screened with TeHV3-seropositive sera from T. *graeca*. Phagemids were excised from the positive bacteriophages, sequenced, and compare with the TeHV3 genome to identify the encoding genes. Six different structural and non-structural proteins have identified as immune relevant. Vero cells where transfected with phagemids of the positive clones, to confirm previous results. TeHV3's proteins expression was assessed by F.A.C.S using T. *graeca* seropositive sera. Of all the six selected clones, only that expressing the partial sequence of the glycoprotein B (gB) showed a positive signal in the F.A.C.S. analysis. This result is consistent with the well-known immunogenicity of gB of other herpesviruses including those infecting humans and with the highly conserved role that gB plays in host-pathogen interaction across species and evolution (Beals et al., 2016).

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New insights

Introduction:

The final objective of this work was to develop a possible oral vaccination against TeHV3. Although different attempts have been made to produce herpesvirus vaccines using gB in humans, better results were obtained with animals. Although complete viral neutralization was never obtained, a sensible reduction in the viral load was evident [1,2]

Basing on our previous results (see section "initial assessment") and literature, gB was the ideal target for this purpose. We would like to generate this vaccination by knocking out all other sequences in the original phagemid we created with the phage display library (see material and methods of initial assessment), leaving only the phagemid and gB sequences. Transformed bacteria cells should be able to express gB on the surface, for this reason the gB sequence has to be glycosylate. Engineered phagemid will be used to transform bacterial cells that could be orally administered to tortoises.

Material and methods:

Sequencing:

Standard Sanger sequencing was used to sequence FMP1 (the phagemid containing the gB sequence). Briefly, two sequencing tubes, containing 0.5 ml of turbo primers (TriLink biotechnologies, Sandiego, CA), one tube reverse and one tube forward, and 1µl of DNA at 70ng/µl, each Mastermix (Thermo Fisher, Switzerland) solution was added to each of the tubes to achieve a total volume of 50µl.

Samples were first amplified with a standard thermocycler according to the following program:

Denaturation	*96°C*	*10 seconds*	*25x*
Anealling	*55°C*	*5 seconds*	
Extensions	*60°C*	*1 minute*	
Elongation hold	*8°C*	*Infinite*	

The PCR product was then centrifuged at 4500 RPM for 40 minutes at +4°C. At the end of centrifugation, the tubes were poured over a blot sheet to be emptied. A second centrifugation was performed at 1000 RPM for 1 minute. The tubes were let to dry in the air for 30 minutes and sent for sequencing. The results of the sequencing were evaluated with SnapGene Viewer (V. 3.3.4).

Enzymatic restriction of FMP1:

5µl of FMP1 was mixed with 1µl DnpI (Promega, Madison, US) and 6µl of enzyme buffer. The reaction mix was incubated at 37°C for two hours, not shaking. The incubation product was loaded on 2% agarose gel to check for the presence of restriction fragments.

PCR knock-out of FMP1 (direct site mutagenesis PCR):

A reaction mix composed of 3µl of 10x Buffer, 0.4µl dNTPs, 0.2µl reverse primed, 0.2µl forward primer, DNA polymerase 1µl, 0.2µl of FMP1, and 25µl of water was prepared.
Primer sequences for gB knock-out were:

- Reverse: AAA TTA ACC CTC ACT AAA GGT TAT AGT TCG GTG CGA ACGT
- Forward: ACG TTC GCA CCG AACTAT AAC CTT TAG TGA GGG TTA ATTT

The following cycling program was set on the thermocycler:

Initial denaturation	*94°C*	*3 minutes*	
Denaturation	*94°C*	*30 seconds*	*18x*
Anealling	*45°C*	*1 minute*	
TestudoExtensions	*68°C*	*9.30 minute*	
Enlongation	*68°C*	*10 minutes*	
Elongation hold	*8°C*	*Infinite*	

PCR products were run on a 1.5% agarose gel.

Agarose gel extraction:

Gel extraction was carried out according to the extraction kit guidelines (MinElute Gel Extraction kit, Qiagen Hombrechtikon, Switzerland).

Bacterial cell transformation:

To perform bacterial cell transformation, XL-1 blue and DH5α E. coli cell strains were used. Both commercially available and home-made competent strains of XL-1 blue and DH5 were used during the transformations. Blue commercial (Chem-Aligent, Switzerland) and home-made XL-1 carried Tetracycline resistance. Ampicillin resistance was carried by both commercial (Thermo Fisher, Switzerland) and home-made DH5α strains.

The transformation procedure was as follows:

- Take cells from -80°C and place them on ice for 5-10 minutes.
- Add one microliter of FMP1 to each tube and incubate on ice for 30 minutes.
- Immerse in warm bath at 42°C for 45 seconds.
- Incubate on ice for 2 minutes.
- Pour the tube content into a Falcon round bottom polypropylene tube (Fisher scientific, Reinach, Switzerland) containing sterile SOC medium.
- Incubate Falcon tube at 37°C for 2 hours.
- Mix 20µl of appropriate antibiotics with 80µl of incubation product.
- Gently spread the content on an LB-agar plate.
- Incubate overnight at 37°CAgarose gel extraction.
- Collect and amplify single colonies.

pET30C⁺ plasmid ligase reaction:

Newly obtained DNA fragments from gel extraction were transferred to a cloning vector called pET30C^{+} (EDM Bioscience, USA).

Two different protocols were used in this reaction:

- 5 microliters solution with 1 microliter ligase mixed with 5 microliters fast ligase buffer. The reaction was incubated for 3 hours at room temperature.
- 5 microlites solution with 1 microliter ligase with 3 microliters buffer. The reaction was carried out overnight with +4C° (fridge) incubation.

Mini- and Midi-preps:

Procedures were carried out as described in the "initial assessment" material and methods section of the previous work.

Results:

Sequencing:

Sequencing produced the following sequence:

TAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGTA
CACTTACTGGTACCCCACCCGGGTGGAAAATCGATGGGCCCGCGGCCGCTCTAG
AAGTACTCTCGAGAAGCTTTTTGAATTCTTTGGATCCACTAGTGTCGACCTGCAG
GCGCGCGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGT
AATCAAGGTCATAGCTGTTTCCTGTGTGAAATGTTATCCGCTCACAATTCCACAC

AATATACGAGCCGGAAGTATAAAGTGTAAGCCTGGGGTGCCTAATGATGAGCTA
ACTCAC AGTAATTGCGGCTAGCGGATCTGACGGTTCACTAAACAGCTCTGCTTA-
TATAGACCTCCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGGCGGAGT
TGTTACGACATTTTGGAAAGTCCCGTTGATTTTGGTGCCAAAACAAACTCCCATT-
GACGTCAATGGGGTGGAGACTTGGAAATCCCCGTGAGTCAAACCGCTATCCACG
CCCATTGATGTACTGCCAAAACCGCATCACCATGGTAATAGCGATGACTAATACG
TAGATGTACTGCCAAGTAGGAAAGTCCCATAAGGTCATGTACTGGGCATAATGC
CAGGCGGGCCATTTACCGTCATTGACGTCAATAGGGGGCGTACTTGGCATATGAT
ACACTTGATGTACTGCCAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACG
TCAATGGAAAGTCCCTATTGGCGTTACTATGGGAACATACGTCATTATTGACGTC
AATGGGCGGGGGTCGTTGGGCGGTCAGCCAGGCGGGCCATTTACCG-
TAAGTTATGTAACGCGGAACTCCATATATGGGCTATGAACTAATGACCCCGTAAT
TGATTACTATTAATAACTAATGCATGGCTTATCTTCAGCGTGTTCCTGATCGAG-
TAGCCACCGAACAAAAACACATGCTGGAGGAGACTAGAAAATGGGTCGGCTCTC
AGTTTATCGAGGAACTCTTGGTTCTGGACGATCAGTGCCGTGAGCCTATTTTTAA
ACCGTATGCGGCTACAAAATTTTCCACGGTGTTTGAACAATCAGAGGAGATTAA
AGATCCTGTGATCAAGGGAAGAACGTTTTTCGGTAGCTTTAAGATGGGTGGAAG
TAAAGGAAAAAGGCGGAGCGTTTGTAAATAAAAACACCGTGTGTTTCTGTAGGA
CTCATTCTCTTAGAGATGGCGAGGGTACGCGAACGCATAGACGTCTTGTATCGGA
CGCCCAGGAATTTGACAGGACATGGTGTCATGGAAGGCAATTATGCCATCGCAT
GCAAAACCATAATGTCTAACTACGGAGAAAATAACCCTCAGGATGTTACCTT-
GGTTCTGAGTGCGTAGGAGGCGATCGATTGGCGGTTATCAATATACCATCCCGTC
ATCGCCTGGACTTTACCAACCCCAAGGTAGGACTTTTGAAGGCCGTGCCTCGCT
TTCCCGACTACTGCGGACTGCTCTGCGAACATCTGGTATCCCACGCAGTCAATGG
ACATTGCCGGGACGACGCATGTGTGGTGTGTCTGGAAGTCGACTATCCTTTCTG-
GAACCACCATTACGCCGTGAAGGATCTGGTCAGGGAAAACTACGGACGACTCTG
GGCCATACCACAGCTGCATTCTCATAGGACCGTCGCCTTAGAGGTAAATGAC-
CTCTGTAAGGCGATGGATGGTGCAGAACATTGGTTTCTCACCGGAATTCCCCTGC
TGGAATATCACCGCTCGGTGAGAGCCTATTTGCAGATACAGGAGATGTGTGAG-
GAACTGAAGAGGTATTATTATGGGATGATTCATTTAACCCATTGGTTCTACGAAC
TGACCATCGAAACGATAATGTTCGTGGACAAGAAGCGCGGTCCTCCGACCAGCA
CCCTGTTAGATCGAATGCCACACACTTGGACCCAGAGGATTATTTCTCTTTTGG-
TAGACATTATCTGTCCGGGATATTTCGTCTACGTATTTTACTCCAACGTGTTCATC
AACGGAGACTTCTTTGTTCACTTATTTGATGAAAAGGAGGTCGGTGTTTAATGGT

CTCGTATATCTTTTTTGTATCGATGATGATGTACGTAGCGGGTATTATTTATGATA
CCATCGATTTCGTAGCTCCTAGGCTGTGGACCTACGCAATTCTAAATATACCC
ATCTGTTACTAAATAAAAAGACATATCAAAAAGCTGTGCTCAGTGTGGTGAT
CTGGGTGGAAAACCTCTGGAAAATACCAAAAAATGCATCTATAAACCGACGAG-
TCATTTATCCGACGAGAATCTGGCCATGGAAGACTCTATTAACCAAATTACGGTG
ATTATGGACAACAGCAGTCATCTATTGATGTTCATCATTCTAGAGGGTTCTCTTCT
GTTAACGTGCTTGGCAAGTTTAGAGTACGCAGATTCGCGCAACAGAAATTAC-
GTTGTGCCGGCGGTACTGGGCAACGCTTTGATTTTTTATTTTGGTTGTTGTGGGG
CCATAGTTTATTATTACTGTTGGCAACACAGACAAGCTAATATCGTTAGATGGG-
TAGTGTTTATCATAAGCCTGATAGCTAACGTAGACATGTGTATCATGGCAGCA
TATTTACCCCAGTACTATTTCATCGAATTGCCTATGTTATTGACCGCAATTTTTG
GATTGTACACCACCCTGGTAATCAGAAAAACCGTGAAAGATCCGTTCGATGCT
GCCGCCGAAAGCGGAATTGGTCCAGATCCGGAACCGTTCTTAGAATTAGTGTC
AGAAACATTCGCGATAGATAGCGAATGCAGAATTTGTTCCATGCTGGAACTTTT
TCGGAAAGCTCGAGTTCAGAAGGTCGAATGGTTTTTAGATTATATCGC GTTATG
TAGGAAATGTCTAGCCGCCCCTTTGTGCGCCACTTCCACCTTCATAACCGCGTTCG
AATTTGCCTACATCATGGAAAAACATTATAGCGAACAGGATGGTATTACCGTAA
TTGGGGTATACAGTAATCGGATGATTTCTGTAAATGACATCCAAAAACATTTTTA
CATACACTGTTGTTTTAAGACCAACGACGGGGTAGTGGGAAAGATCGGGCTAAC-
CAATCAACGCACACGGTAGTTCAGGTGGCCCAACCCACAACGTTCGCACCGAAC
TATAATACTATATAACTTATTATAATATAATAAGAAGGTGCGAACGCTG-
GATCGAGTCAGTTCGTACAGCAGCTTTTCTCCAAATTCAATATACGTACGATA
AACTGCAAGCTCATAACAACGCCATGTTCTCTCGTATCGTATACGCTTGGTGC
GAGTTACAAAATCGAGACATAACCATGTGGGAACAATTAAATAAAATAAATC
CCTCGGCGGTCATGTCCGCCATAATGAAGCGTCGCGTTTCGGCCAAAAAATTGG-
GAGACGTGGTGGCCGTTTCCGACTGTTTAACTATTCCCAGGAACAATGTCCGTCT
CATCAATTCCATGTATATCACCAAGGGAAATTTG TAGGTAGCGGTGTGTGTTATA
GCCGTCCGTTGGTAGTTTATACATTCGGAAACAGTACCGAAAGCCTCTATGG
TCATCTGGGAGAAAATAATGAAATCATCCCTTACGTGGGATTGACGGAAAACT
GCGAGCCAAAATCCAGGAAACTGTTTCTTTACGAGAACGCATATATGCTGTAT
GAGAACTATAATTTTGTTAGGATGGTTCCATTAACCGACATCCCAGAAGTAAGCA
CGTATATCAATCTCGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCCAGCT
TTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCAAGGTCATAGCTG
TTTCCTGTACGCCAGGGTTTTCCCAGTCACGA CGTTGTAAAA CGACGGCCAGTG

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TTCAGGTTCA GG GGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTC-
TACAAATGTGGTATGGCTGATTATGATCATGAACAGACTGTGAGGACTGAGGG
GCCTGAAATGA GCCTTGGGACTGTGAATCTAAAATACACAAACAATTAGAATC
AGTAGTTTAACA CATTATACACTTAAAAATTGGATCTCCATTCGCCATTCAGGCT
GCGCAACTGTT GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCA
GCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGG
GTTTTCCCAGTCACGACGTT.

Evaluation with SnapGene© demonstrated that the gB sequence was antisense compared to the plasmid ORI, as demonstrated in Figure 1.

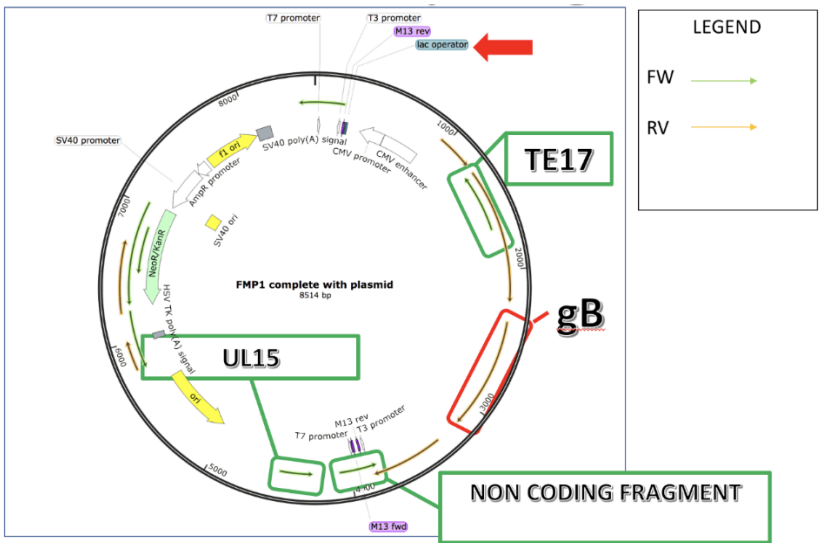

Figure 1: FMP1 sequencing results. gB (red) in antisense compared to the phagemid ORI. However, other two forward sequences are present in the scheme (green), TE17 and UL15. Legend: FW = forward and RV= reverse.

Enzymatic restriction of FMP1:

DpnI enzymatic cutting produced different fragments among which gB, TE17 and UL15 were present as shown in Figure 2 (SnapGene Viewer). Fragments were run in a 2% agarose gel and extracted according to migration height, basing on the DNA sequence molecular weight.

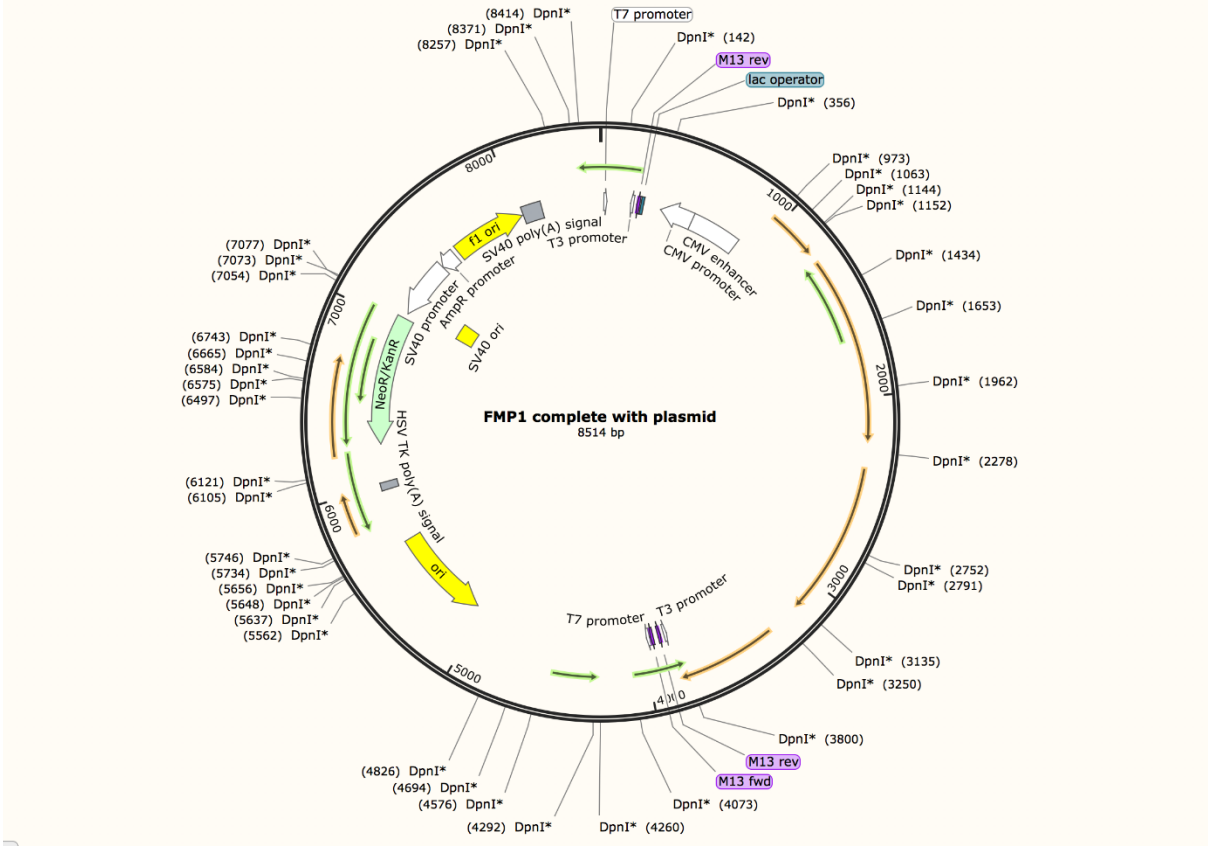

Figure 2: Fragments compatible with gB, TE17, and UL15 size were extracted from the original phagemid and inserted in new plasmids, which were used to transform bacterial cells (SanpGene viewer V4.0.3).

PCR knock-out of FMP1 (direct site mutagenesis PCR):

Like the enzymatic restrictions products, PCR products resulting from direct site mutagenesis were also used to transform bacterial cells.

Ligase:

gB, TE17, and UL15 fragments obtained from FMP1 were inserted in the new cloning vector using a standard ligase procedure. Bacteria were than transformed using the procedure described in material and methods.

Bacterial cell transformation

Although numerus attempts were made to transform bacterial cells with FMP1 knock-out, no viable bacterial cells were obtained.

To increase the transformation chance, fragments were transferred into a new cloning vector (pET30C^{+}). Using this cloning vector, some viable colonies were obtained (Figure 2). Although colonies were identified on LB-ampicillin plates, multiple attempts at mini- and midi-prep extraction of the plasmids were unsuccessful.

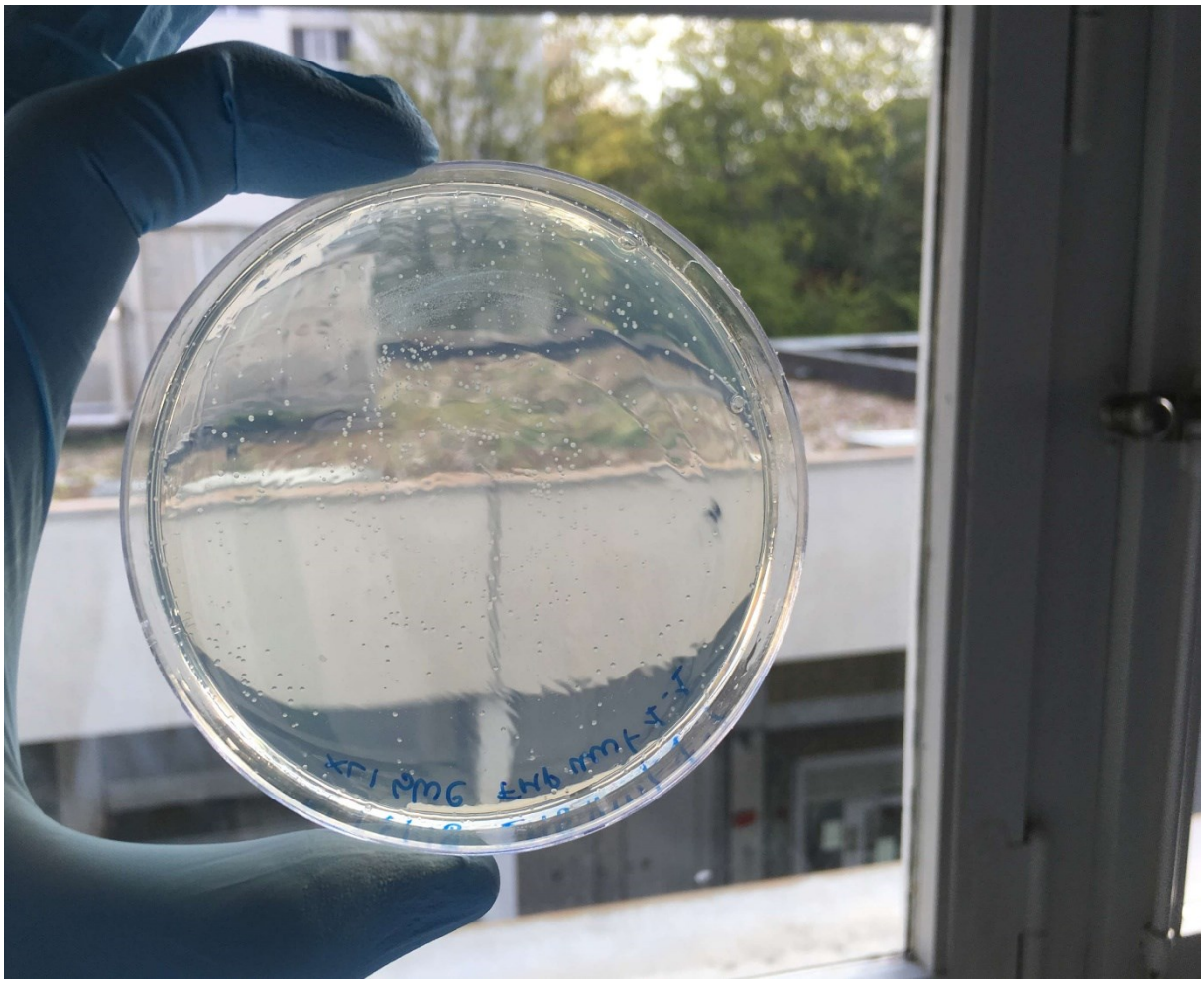

Figure 2: LB-Ampicillin plate with multiple E. coli colonies transformed with pET30C+.

Discussion:

The sequencing results demonstrate that gB was actually antisense and could not be expressed during eukaryotic cell transformation. However, two other sequences were consensus to the plasmid origin of replication (ORI) and might be responsible for the results obtained in the "initial assessment" part. Still, the immunogenic role of gB could not be excluded. To better complement the immunogenic nature of gB, TE 17 and UL15 we knocked out all the genes individually. No viable colonies were obtained with enzymatic restriction of PCR mutagenesis using FMP1 as template. Taking the overall size of the plasmid into account, we considered that this might be the cause of the unsuccessful bacterial transformation. To reduce the size of the plasmid we opted to use pET30C^{+} as cloning vector (Figure 3).

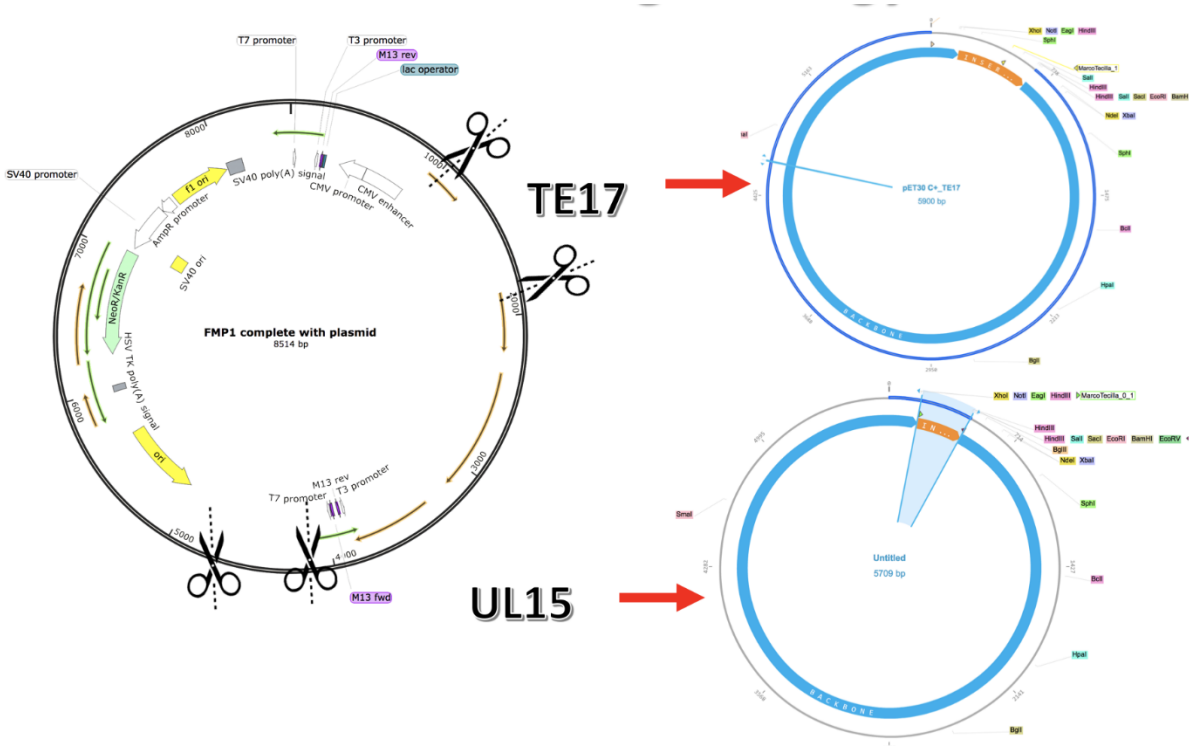

Figure 3: Schematic representation of TE17 and UL15 extraction from FMP1 and insertion into the new cloning vector pET30C+.

With pET30C^{+} we were able to obtain viable colonies. Although the presence of viable colonies, both mini- and midi-prep techniques failed to retrieve transformed pET30C^{+} from the selected colonies.

Despite numerous attempts we were never able to understand why colonies without the insert were able to grow on antibiotics with added LB. To increase selectivity, we also tried to increase the amount of antibiotics, still having the same issue.

We are still working on troubleshooting the problem.

References:

1. Kemble G, Spaete R. Herpes simplex vaccines. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press; 2007. Available: http://www.ncbi.nlm.nih.gov/books/NBK47451/

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CORRESPONDING AUTHOR

Marco Tecilla
marco.tecilla@unimi.it

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UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE PER LA SALUTE, LA PRODUZIONE ANIMALE E LA SICUREZZA ALIMENTARE

Characterization of the immune response against Testudinid herpesvirus 3: new insight

Marco Tecilla[1*], Paola Roccabianca[1], Paola Pilo[2], Francesco Origgi[2,3]

[1]*University of Milan, Department of Veterinary Medicine, Italy*
[2]*University of Bern, Institute of Veterinary Bacteriology, Switzerland*
[3]*University of Bern, Centre for Fish and Wildlife Health, Switzerland*

Abstract

Testudinid herpesvirus 3 (TeHV3) is one of the most lethal viral agents in tortoises worldwide. Although TeHV3 have been extensively studied, only little information is available about host-pathogen interaction. TeHV3 infections in different species of the genus *Testudo* correlate with various lesions profiles, disease severity and clinical outcome, suggesting the existence of a complex host-pathogen interaction. This might reflect a possible viral-host coevolution (Origgi, 2012).

To study the host-pathogen interaction, we previously screened 5.000 clones from a bacteriophage library obtained from the TeHV3 genomic DNA using *Testudo graeca* seropositive sera. Of the six detected positive clones, only one was confirmed by F.A.C.S. Selected clone was determined to be a concatamer of different TeHV3 genomic fragments including the partial sequence of TE17, UL15, Major capsid protein (MCP), and Glycoprotein B (gB) genes. After complete sequencing of the selected clone, the MCP and the gB were antisenses compared to the phagemid promoter.

In order to assess which of the gene fragments among TE17 and UL15 was encoding for the antigenic determinant that was recognized by the anti-TeHV3 tortoise sera, distinct approaches were followed. TE-17 and UL15 fragments were knock out from the original phagemid using the following approaches: a) directed-site mutagenesis, b) molecular cloning, and c) restriction enzymes cloning. All the modified constructs were cloned in two different E. coli cloning vectors (D5α and XL 1-Blue).

Transformation of competent cells with the constructs described above did not yield any viable bacteria.

Among the different aspects might have influenced transformation success rate, construct size was probably the most relevant (about 9Kb). Furthermore, we could not entirely exclude that genomic DNA editing might have induced mutations in the construct sequence causing toxic effects on the host bacterial cell. Cloning of TE-17 and UL15 gene fragments into different prokaryotic expression vectors is currently under way.

References

Origgi, F.C., 2012. Testudinid Herpesviruses: A Review. Journal of Herpetological Medicine and Surgery. 22, 42–54.

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Conclusions

The present work aimed to:

1) Identify the presence of *Testudinid herpesvirus* in Italy
2) Genetically characterize *Testudinid herpesvirus* 3
3) Start to describe the host immune response against *Testudinid herpesvirus*

The chelonian herpesvirus represents a threat to captive bred and wildlife turtles and tortoises worldwide.

Although many studies have attempted to develop detection methods and understand the pathogenesis of these viruses, important information such as the distribution, prevalence, incidence, viral biology, and host-pathogen interaction is missing or incomplete.

According to the literature, worldwide distributed chelonian herpes viruses are the Chelonian herpesvirus 5 in *Caretta caretta* and *Chelonya mydas*, *Testudinid herpesvirus* 2 in American desert tortoises, and *Testudinid herpesvirus* 3 in Mediterranean tortoises, but the specific host-pathogen distribution is limited.

In Europe, turtles and tortoises are common companion animals. As a consequence, tortoises of different breeds are extensively bred and traded within Italy or with other European countries and constitute a relevant economic income.

Although chelonian herpesviruses are anecdotally reported as a common cause of death in chelonians, evidence of mortality related to herpesviral infections, correlated with disease prevalence and incidence, is only reported in a few studies, among which only two were performed in Europe.

In the present study, numerous terrestrial tortoises and freshwater turtles from northern Italy were examined to identify the presence of chelonian herpesviruses in Italy, in both a prospective and retrospective way. All of the evaluated TeHV-positive cases except one were positive for *Testudinid herpesvirus* 3, which is the most common chelonian herpesvirus in Italy. The only TeHV-positive animal that was negative for TeHV3 was a retrospective *Testudo horsfieldii*, which was positive for TeHV1.

The identification of the TeHV3 genotype in Italy is in agreement with previous studies performed in the United Kingdom and Spain; in addition, the prevalence of this virus as evaluated via PCR was similar. This suggests that, throughout history, there might have been a homogenous distribution of the virus in Europe that was probably secondary to the reptile trade and the release of pet reptiles into the wild.

The serological prevalence of TeHV3 in Vanzago's tortoises was extremely high and could not be compared to any other prevalence evaluated in other studies. According to our opinion,

the prevalence was so high because of the management of the animals. Turtles and tortoises were introduced into the Oasis without any previous clinical examination, quarantine, or TeHV testing. Furthermore, even though no clinical abnormalities were detected during the periodic check-ups, quarantine of the affected animals was not possible due to the limited space available; this promoted the spread of the virus.

In Italy, wildlife rescue centers are nationally recognized and funded structures that are actively involved in wildlife conservation and reintroduction as well as in monitoring and controlling exotic and allochthonous species. Despite the obvious social relevance of wildlife rescue centers, their low budgets, limited spaces, lack of quarantine areas, and overcrowding makes the management of these structures extremely complicated. In addition to all of these problems, wildlife rescue centers are officially appointed as keepers of exotic or wild animals seized due to being owned illegally, which cannot be rejected or euthanized even if diseased. The present study raises the question of whether the management of these structures might represent a threat for infectious diseases diffusing into wildlife populations after the reintroduction of rescued animals into the wild.

In this study we also compared the effectiveness of anatomical pathology and histopathology in detecting the presence of the virus. In our caseload, and contrary to other studies, the efficacy of gross and histopathology in detecting TeHVs associated lesions was low, particularly in samples from Vanzago's Oasis. In our opinion, the sensitivity and specificity of our results was influenced by the typology of the samples we included in the study. The presence of biopsy, "necropsy in a bottle," and autolytic bodies from Vanzago represented a different caseload compared to studies available in literature, where only necropsies of fresh or minimal autolytic bodies were considered for the anatomopathological evaluation.

The inclusion of different typology cases allowed us to arrive at two conclusions:

1) Biopsy and "necropsy in a bottle" are samples frequently submitted from veterinarians to pathology diagnostic services. Based on our findings, the diagnostic relevance of the samples mentioned above relies almost entirely on the ability of the referral veterinarian in detecting the right specimens to submit. Furthermore, gross observations of the referral veterinarian are frequently missing, which does not allow the pathologist to correlate gross and histological findings.
2) Most of the bodies submitted from Vanzago's Oasis were characterized by the presence of mild to severe autolytic changes. TeHV3-associated mortality occurs mostly during hibernation or, less frequently, estivation, while animals are dormant in underground burrows and physiologically immunosuppressed. Hibernation length is variable among tortoises, even in the same enclosure, and correlates with environmental conditions, animal health status, age, and breed. Due to the variable length of hibernation, Vanzago's Oasis staff was instructed by the management to wait one to two weeks after the first turtle's awakening to recover the bodies. Although autolysis was initially an unwanted circumstance, we believe that Vanzago's animals' conditions might represent a more realistic model of how the disease occurs in nature, suggesting that the use of anatomical pathology in wildlife outbreak screenings might be of limited help.

In both the prospective and retrospective samples, both animals with no lesions that were PCR positive and animals with classically TeHV3-associated lesions that were repeatedly negative to the PCR were found. This finding suggests that molecular tests (e.g. ELISA, PCR, and viral isolation) should always be implemented to corroborate anatomopathological findings.

TeHV3 genomic sequencing and comparison among different strains collected in Italy, Switzerland, and the USA allowed us for the first time to describe the viral genome and include this virus in the subfamily *Alphaherpesvirinae* proposed genus *Scutavirus*. Phylogenetic analysis of the viral genome based on the gB and DNA-polymerase sequences showed that the virus has many characteristics with the Chelonian herpesvirus 5 (ChHV5) and ChHV6 and, to a lesser extent, to the human simplex virus and the varicella-zoster virus.

Genomic differences between TeHV3 and ChHV5 include genes encoding for enzymes (UL13, UL50, UL40, UL45, UL 54) or structural proteins (UL44, encoding for the gC), which are present in TeHV3 and may play a key role in host immune evasion. Furthermore, the presence and expression of different genes might explain the existence of diverse lesion patterns induced by the viruses: necrotizing lesions in tortoises infected with TeHV3 and proliferative lesions of a neoplastic nature in sea turtles infected with ChHV5.

The comparison among TeHV3 strains from different parts of the world allows subdividing TeHV3 into two genogroups (A and B) that correlate with different lesional profiles in animals. A third genogroup (C), genetically inbetween the first two, was also detected in a single individual from Switzerland, but the bootstrap results obtained were not strong enough to confirm the presence.

While genogroup A was detected in the USA, Italy, and Switzerland, genogroups B and C were detected only in very restricted areas of the northwestern part of Switzerland, suggesting that spontaneous mutations with genetic segregation might have played a role in the development of these TeHV3 genogroups. Excluding genogroup C, which was detected in a single animal, genogroup B detection in Switzerland was identified during a timespan of eight years, always in the same area, preceded and followed by genogroup A outbreaks. It would be of interest to conduct a retrospective survey of the respective countries surrounding the geographical regions of genogroups' B and C outbreaks (France for genogroup B and Germany for genogroup C) to compare the genotype distribution in the same years.

Viral genome sequencing allowed us to develop a phage display library to start detecting the host antigenic response against TeHV3.

Due to the limited knowledge available on reptile immunology, strict standardization of the experimental condition was necessary to gain the most reliable information, so we decided to focus only on the American TeHV3 strain and to use hyper-immune *Testudo graeca* sera collected during the first transmission study performed on TeHV3 by Origgi et al. in 2004. Five clones were selected via immunoblot, each of them containing genes or a part of gene encoding for a range of 1-6 proteins. Among the initially identified clones, only the ones comprising gB, TE17, and Ul15 sequences were also positive at F.A.C.S. analysis. Based on the information available in the literature on the relevant immunological proteins of *Alphaherpesvirus* in other species, gB was the most promising antigenic protein targeted by the host (*Testudo gaeca*) immune response.

Unfortunately, sequencing of the clone returned that the gB sequence was antisense compared to the ORI of the phagemid.

Because two sequences other than gB were present in the phagemid, DNA was extracted and reduced to fragments corresponding to each of the coding DNA sequences present in the phagemid. New fragments were then included in plasmids and expressed in bacterial cloning vectors.

Unfortunately, despite the numerous attempts and various molecular biology techniques used to express TE17, UL15, and gB, we did not succeed.

This is the first time that host-pathogen interaction is investigated in reptiles. Although unable to identify the most antigenic protein involved in host immune system activation, we demonstrated that:

1) Reptiles' immune system is capable of discerning specific proteins among all viral proteins contained in the virus.
2) At least two proteins seem to be immunorelevant in the host immune response against TeHV3. Among those proteins, TE17 sencode for a tegument protein might play a relevant role in early immune response against the virus.
3) The other four clones we detected with the immunoblot technique might represent tortoise IgY antibodies cross-reacting with multiple epitopes, and still might represent a defense mechanism for the host.

Future perspective

Immunology in reptiles is an expanding field that interconnects with new disciplines such as eco-immunology and eco-toxicology, which are growing in importance.
Knowledge of reptiles' immune system might open new research frontiers, including the use of reptiles as research animals or in ecological and pollution surveillance plans.
Furthermore, from a biodiversity perspective, understanding reptiles' immunology will provide new therapeutical approaches to prevent the extinction of these animals, secondary to the diffusion of new and old diseases such as TeHV3.
In the future, we will have to characterize the exact proteins involved in boosting the host immune system against TeHV3 to try to develop a vaccine against the virus. Moreover, we will try to describe the immune response in other tortoises (*Testudo hermanni* and *T. marginata*) to see if there are differences, and then try to correlate those differences with a possible host-pathogen co-evolution.
Once this information is obtained, we would like to create a snap ELISA test able to identify TeHV3 genogroups efficiently and cost effectively.
With this tool it will be possible to set up a network of veterinarians worldwide to establish viral genogroup distributions and try to create genogroup-specific therapeutical approaches.

Publications outside the PhD research project:

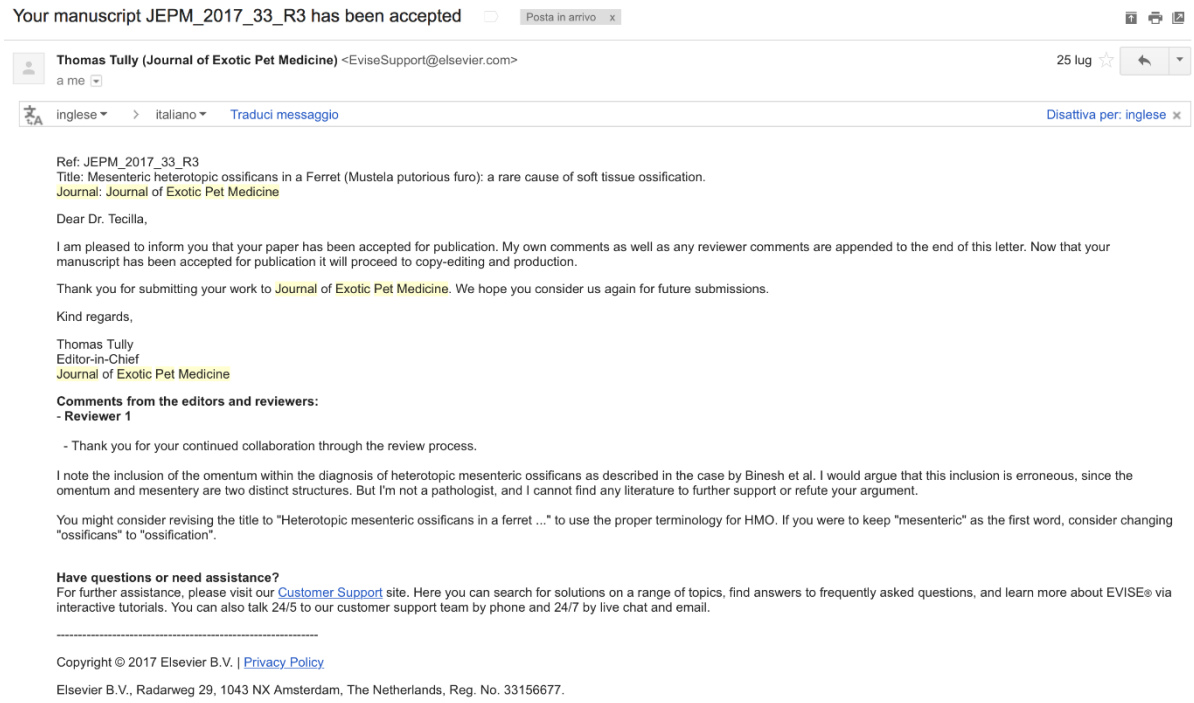
Indexed publications:

1: Avallone G, Forlani A, **Tecilla M**, Riccardi E, Belluco S, Santagostino SF, Grilli G, Khadivi K, Roccabianca P. Neoplastic diseases in the domestic ferret (Mustela putorius furo) in Italy: classification and tissue distribution of 856 cases (2000-2010). BMC Vet Res. 2016 Dec 5;12(1):275. PubMed PMID: 27919252; PubMed Central PMCID: PMC5139086.

2: Bel L, **Tecilla M**, Borza G, Pestean C, Purdoiu R, Ober C, Oana L, Taulescu M. Diagnosis and surgical management of malignant ovarian teratoma in a green iguana (Iguana iguana). BMC Vet Res. 2016 Jul 19;12(1):144. doi: 10.1186/s12917-016-0773-x. PubMed PMID: 27435282; PubMed Central PMCID: PMC4952068.

3: Gianella P, **Tecilla M**, Bellino C, Buracco R, Martano M, Zanatta R, Cagnasso A, D'angelo A. An unusual case of intestinal leiomyositis in a Bernese mountain dog. Schweiz Arch Tierheilkd. 2015 Oct;157(10):563-7. PubMed PMID: 26897772.

4: **Tecilla M**, Bielli M, Roccabianca P. Mesenteric heterotopic ossificans in a Ferret (Mustela putorious furo): a rare cause of soft tissue ossification. Journal: Journal of Exotic Pet Medicine. Under publication (See acceptance letter below).



Your manuscript JEPM_2017_33_R3 has been accepted Posta in arrivo x

Thomas Tully (Journal of Exotic Pet Medicine) <EviseSupport@elsevier.com> 25 lug

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Ref: JEPM_2017_33_R3
Title: Mesenteric heterotopic ossificans in a Ferret (Mustela putorious furo): a rare cause of soft tissue ossification.
Journal: Journal of Exotic Pet Medicine

Dear Dr. Tecilla,

I am pleased to inform you that your paper has been accepted for publication. My own comments as well as any reviewer comments are appended to the end of this letter. Now that your manuscript has been accepted for publication it will proceed to copy-editing and production.

Thank you for submitting your work to Journal of Exotic Pet Medicine. We hope you consider us again for future submissions.

Kind regards,

Thomas Tully
Editor-in-Chief
Journal of Exotic Pet Medicine

Comments from the editors and reviewers:
- Reviewer 1

- Thank you for your continued collaboration through the review process.

I note the inclusion of the omentum within the diagnosis of heterotopic mesenteric ossificans as described in the case by Binesh et al. I would argue that this inclusion is erroneous, since the omentum and mesentery are two distinct structures. But I'm not a pathologist, and I cannot find any literature to further support or refute your argument.

You might consider revising the title to "Heterotopic mesenteric ossificans in a ferret ..." to use the proper terminology for HMO. If you were to keep "mesenteric" as the first word, consider changing "ossificans" to "ossification".

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Non-indexed publications:

1: Claudia Manno, Damer Blake, Gabriele Ghisleni, **Marco Tecilla**, Giusi Macaluso, Roberto Puleio, Vinenzo Monteverde, Guido R. Loira. *Cardiac filariosis in migratory Mute swans (Cygnus olor) in Sicily. International journal of Health, Animal science and Food Safety; DOI: https://doi.org/10.13130/2283-3927/6796*